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ABSTRACT

This report summarizes research supported by the EPA Health Effects Research Laboratory at Research Triangle Park, NC, under the Federal Interagency Energy/Environment R & D Program. The EPA has had the lead responibility for the planning, coordination and implementation of this program since fiscal year 1975.

Projects reported in this document are grouped under one of four major research areas. The first area is identification of hazardous agents associated with non-nuclear energy technologies. These projects involved the development of qualitative methods for the identification of hazardous materials. The second area is development of more rapid and sensitive methods to evaluate dose to man. These projects focused on the development of quantitative methods for measuring degree of toxicity of various pollutants. The third area is determination of the metabolism and fate of hazardous agents associated with energy technologies. These projects involved determination of the physiological activities of several known carcinogens. The fourth research area is evaluation of hazards to man. In addition to studies of the effects of certain pollutants on humans, several of the projects concerned preparation of standard pollutant samples for use in future studies to increase the comparability of results.

A list of additional studies funded under this program is included.

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Project Status Report

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January 1979

INTERAGENCY PROGRAM IN ENERGY-RELATED HEALTH AND ENVIRONMENTAL EFFECTS RESEARCH

Project Status Report

Health Effects Research Laboratory
Office of Health and Ecological Effects
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, N.C. 27711

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
OFFICE OF HEALTH AND ECOLOGICAL EFFECTS
HEALTH EFFECTS RESEARCH LABORATORY
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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

When energy and material resources are extracted, processed, converted and used, waste products are emitted which may have a significant impact upon public health and the environment. A highly integrated, multidisciplinary research and development effort is required to assess this impact. The research projects reported on in this document constitute this Laboratory's input to this Interagency Program.

F. G. Hueter, Ph. D.
Acting Director,
Health Effects Research Laboratory

ABSTRACT

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Projects reported in this document are grouped under one of four major research areas. The first area is identification of hazardous agents associated with non-nuclear energy technologies. These projects involved the development of qualitative methods for the identification of hazardous materials. The second area is development of more rapid and sensitive methods to evaluate dose to man. These projects focused on the development of quantitative methods for measuring degree of toxicity of various pollutants. The third area is determination of the metabolism and fate of hazardous agents associated with energy technologies. These projects involved determination of the physiological activities of several known carcinogens. The fourth research area is evaluation of hazards to man. In addition to studies of the effects of certain pollutants on humans, several of the projects concerned preparation of standard pollutant samples for use in future studies to increase the comparability of results.

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SECTION I

IDENTIFY HAZARDOUS AGENTS ASSOCIATED WITH NON-NUCLEAR ENERGY TECHNOLOGIES

A. TASK TITLE:

Development of Methods for Determination of Carcinogenesis by Bacterial Mutagenesis Employing Crude Material from Alternate Energy Sources.

HERL/RTP TASK NO: 8151

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Summary

Ames originally developed an *in vitro* mutagenic screening method using special strains of *Salmonella typhimurium*. These strains have a histidine-negative genome and include other genetic factors that render them specifically sensitive to chemical mutation. Mutation causes them to revert to a histidine-positive genome, so that the presence of a mutagenic agent can be determined by counting the number of colonies that appear when bacteria are inoculated on a nutrient medium that lacks histidine.

The original system has been employed primarily to assay pure compounds or fairly simple mixtures. The technique has been modified under this project in order to effectively use it to analyze crude mixtures or organic compounds which may occur in coal and oil shale conversion processes; and as a biological screening technique. Significant modifications include:

1. development of a liquid culture method as opposed to the conventional plate method;
2. development of improved methodology for the analysis of mutagenic urinary metabolites of carcinogens based on the liquid/culture method;
3. development of techniques for producing dose response curves of mutagenic activity, and;
4. development of a procedure for comparing the "relative mutagenic activity: of different samples.

The modified techniques have been tested and employed on analyses of samples of coal liquification products, atmospheric particulate samples from Los Angeles and Pittsburgh, and human urine samples.

Scope and Objective

Determine the carcinogenicity of crude alternate energy source effluents and products by means of *in vitro* systems previously developed in this project; includes whole animal and other means of detecting carcinogenic metabolites in the urine for eventual application of clinical and population studies of groups exposed to various carcinogenic hazards.

Background and Approach

Most carcinogenic compounds are not mutagenic toward *Salmonella* unless they are converted to metabolic intermediates by the action of suitable tissue microsomal preparations. The *Salmonella* method can generally distinguish between presumptive carcinogens (compounds which have been shown to cause cancer in laboratory animals, but which *may* or *may not* cause cancer in people) and noncarcinogens. However, the *Salmonella* mutagenesis systems now are employed primarily to assay pure compounds or relatively simple mixtures. Certain modifications of the technique are necessary in order to effectively use it to analyse crude mixtures of organic compounds, particularly as they occur in the various stages of coal conversion processes and shale oil processing. The initial approach as outlined below has been modified during the course of the project, as indicated under Research Accomplished.

In the conventional *Salmonella* test procedure, the sample is mixed with the liver homogenate and the bacterial inoculum and spread over the entire surface area of the test plate. In one modified procedure, aliquots of extracts from the crude sample are evaporated from a suitable solvent onto small pieces (1 cm diameter) of sterile filter paper. The microsomal preparations and the bacterial inoculum are mixed and spread over the agar plate and the filter discs carefully placed on the top of the agar surface. The appearance of colonies of revertant mutants in the medium, in statistically significant numbers (compared to controls discs which lack the test compound) surrounding the disc, indicate the presence of presumptive carcinogens. Because this procedure concentrates the sample in the filter paper, it can enhance the overall sensitivity of the method, and also give some indication of the presence of active substances which differ in their rates of diffusion.

In the preliminary screening, all samples are tested against a maximum of five different strains of *Salmonella typhimurium*, including TA 1535, Ta 100, TA 1537, TA 1538, and TA 98, and any additional strains of superior characteristics that may become available during the course of the work. A minimum of three different rat liver microsomal preparations (induced by sodium phenobarbital, PCB, and where possible, the crude sample itself) which differ in their inducing agent are used for each test. The microsomal preparations are isolated by standard biochemical methods.

The standard bacteriological plates contain a suitable agar nutrient medium that lacks histidine. These plates are prepared in duplicate, together with duplicate control plates (identical with the above but lacking the crude test substance on the filter paper disc). An additional control consists of a plate inoculated with a mixture of bacteria and the substance being tested--i.e., with no added microsomal preparations. After 48 hours incubation at 37°C, counts of revertant colonies are made in a circular zone surrounding the disc and compared with those of the control discs to determine mutagenic activity.

If the sample shows mutagenic activity in this test, it is further tested to determine dose-response relationships by the standard Salmonella test. The strain-microsomal combinations which yielded the highest mutagenic activity in the paper disk method are selected and retested by the standard Salmonella test at a series of different concentrations of the crude mixture to establish dose-response relationships.

In addition to the direct Salmonella test of the whole crude sample described above, each of the samples is fractionated by thin layer chromatography. Then, strips of the chromatographic fractions are placed on plates seeded with the bacterial strains and the microsomal preparations. The test plates are incubated for 48 hours at 37°C and zones of revertant colonies surrounding the paper strips are located (by comparison with appropriate control paper strips with no test compound). Plots of the number of colonies as a function of distance along the strip are prepared when appropriate.

Each of the crude test samples is subjected to thin layer chromatography using at least two different solvent systems, and is tested against the same five different strains of Salmonella and three different microsomal preparations. Each of the tests is made in duplicate together with duplicate control plates (which lack the crude test substance on the TLC)--and also an additional control plate which lacks the microsomal preparation.

If the TLC paper strip shows zones of mutagenic activity, the corresponding zone from the main chromatogram is cut out and extracted using an appropriate solvent such as benzene, hexane, or chloroform. The extract is taken to dryness and analyses undertaken to identify the chemical component responsible for the mutagenic activity. Studies to identify mutagenically active compounds include one or more of the following physico-chemical methods: U.V.; NMR; mass spectrometry; ESR hyperfine labelling and comparison of chromatographic properties with known standards.

The results of tests on mixed crude samples are compared with the results of bacterial mutagenesis tests on the separate fractions of the thin layer chromatogram to determine the effect of synergistic interactions and interference among the substances present in the mixed samples.

The Salmonella test relies on the now well established idea that while some carcinogenic substances appear to be the proximately active agents which directly induce the process of carcinogenesis, many such substances must be converted metabolically before they are active, proximate carcinogens. Substances of the latter type are mutagenic in the Salmonella test system only after being converted to active metabolites by a microsomal preparation which is included in the in vitro test system for that purpose. In the standard Salmonella test this activation system consists of the 9,000-g supernate of a rat liver homogenate, to which has been added NADPH and glucose-6-phosphate, as a means of supplying an electron source for the oxidative process.

It is clear, however, that the activity of the microsomal preparation is much more complicated than a simple conversion of a carcinogenic substance into a given active metabolite. In previous tests it was found that some presumptive carcinogens are "false negatives." That is, they apparently are not converted into active metabolites by the microsomal preparation as it is now used. These observations indicate that our knowledge of the activation process, as it occurs in the crude microsomal system, is rather superficial, and needs to be refined in order to maximize the sensitivity and reliability of the Salmonella test system.

One way to improve the activation process is to administer the test compound to whole animals and to determine the presence of carcinogenic metabolites in the body fluids of the animal, such as urine, blood, or bile. Studies have shown that mutagenic metabolites appear in the urine of laboratory animals that have been fed two of the carcinogens that were "false negatives" in the standard in vitro Salmonella test (N,N-dimethylaminoazobenzene and 1,2,5,6-dibenzathracene). The approach then will be to administer the crude test sample to laboratory animals (either by injection or feeding) and to test their urine by means of the Salmonella method. If any mutagenically active metabolites are formed, they will be fractionated by the thin layer chromatographic procedure. Attempts are made to identify the active compound(s). Since such metabolites may not always be excreted in urine, it may be necessary to also analyse blood or bile samples for mutagenic activity.

Research Accomplished

Using the Ames test as a basis, a liquid test system was developed in which not only mutation, but the actual replication of mutated bacterial cells takes place in a liquid culture medium. After a period of incubation the numbers of non-revertant and mutant (revertant) cells are then determined by spreading aliquots of the incubated culture on agar plates containing an excess of histidine and no histidine, respectively. The effectiveness of the new liquid method was compared with a procedure in which the urine is hydrolyzed with B-glucuronidase to free metabolites from their conjugates; it is then extracted with ether or a mixture of benzene and isopropanol and the extract, after being reduced to dryness, is taken up in DMSO and aliquots are tested for mutagenicity by the standard plate method. The extraction step is essential in order to remove histidine (often present in urine) which would otherwise support the growth of non-revertant bacteria and thus obscure the counts of revertant bacteria on the test plate. The liquid method obviates the need for removing histidine, so that the entire process can be compressed into a single step, in which urine together with B-glucuronidase is added to an incubation medium containing an appropriate Salmonella strain and microsomes (if desired).

In order to validate this procedure, the capability of the liquid and the conventional plate method was compared in detecting mutagenic material in benzene-isopropanol extracts of urine. The results were found to be generally similar with both methods. In extracts of urine from AAF-fed rats, some activity is observed by the plate method in the absence of microsomes (indicative of inherently active mutagens); in this circumstance the liquid method appears to yield no activity. Much more activity is observed by both methods when microsomes are present (indicative of activatable mutagens). If the sample is first treated with B-glucuronidase the activity is considerably enhanced.

The ultimate aim of the urine test is to provide a rapid and inexpensive method of analyzing human urine in order to determine whether an individual is being exposed to (and metabolizing) an environmental carcinogen. The original urine technique (i.e., based on conventional plate tests of urine extracts) was applied to a group of human urine samples, and showed that most samples exhibit no activity, with a small percentage of samples exhibiting a positive effect. In an initial test of the new liquid method on human urine, essentially all samples were negative with respect to mutagenic activity. The variation among the samples was about the same in the two methods.

The following three procedures developed under this project were tested on human samples from farmers with one group using pesticides and the other not.

1. Whole urine/liquid method: In this method a sample of urine (sterilized by passage through a millipore filter) is added to a mixture consisting of nutrient broth, an inoculum of a suitable Salmonella strain, a microsome preparation, and a solution of B-glucuronidase. (The B-glucuronidase hydrolyzes conjugates of mutagenic components, releasing them in an active form. The microsome preparation oxidizes those mutagens which require activation to form active metabolites.) The entire mixture is incubated at 37°C for 16 hours. At that time aliquots are removed and inoculated on standard histidine-free culture plates. Revertant colonies (i.e., those derived from cells which have mutated to a histidine-positive genome) that are present in the incubated culture will grow in these plates, and their number is determined by counting duplicate plates.

2. Urine extract/liquid method: In this procedure B-glucuronidase is added to the urine sample adjusted to pH 7.0 which is then incubated at 37°C for 18 hours to hydrolyze conjugates. The sample (150 ml urine) is then extracted twice with 100 ml aliquots of benzene:isopropanol (80:20). The aqueous residue is then adjusted to pH 2.5 and refluzed for 15 minutes to hydrolyze esters of potentially mutagenic constituents, followed (after cooling) by extraction in benzene: isopropanol, as above. The remaining aqueous fraction is then adjusted to pH 11.0 and again extracted with benzene: isopropanol. Each of the benzene:isopropanol extracts (obtained at pH 7.0, 2.5 and 11.0) was then dried in a rotary vacuum dryer and the residues taken up in DMSO. The residues were then added to liquid cultures and treated as described under (1) above. Aliquots representing 1, 5

and 10 ml or urine were tested in this way, both in the presence and absence of microsomes.

3. Urine extract/plate method: In this procedure urine extracts were prepared at pH 7.0, 2.5 and 11.0 as described under (2) above. Each extract, taken up in DMSO, and aliquots representing 5 ml of urine were then added to standard test plates in the usual way and the number of revertant colonies determined. These tests were carried out both in the presence and absence of microsomes.

With the exception of a few samples, all results were negative, based upon the "mutagenic activity ratio" described below. There was considerable variation in the negative values for duplicate samples, probably due to random error in the techniques. Recently Ames, et al., reported a method for concentrating mutagens from human urine about 200-fold for subsequent assay in the Salmonella mutagenesis test. With this procedure he was able to demonstrate the presence of mutagens in the urine of cigarette smokers. In this method the urine is put through a 1.5 cm^3 bed volume XAD-2 column and the absorbed material is then eluted with a few milliliters of acetone. The acetone is taken to dryness and the residue is dissolved in DMSO. Preliminary tests to evaluate this method were conducted under this project.

Urine samples from five males (two nonsmokers and three smokers) were analyzed. These tests were carried out against TA 1538 with and without the presence of microsomes. In each test a 25 ml equivalent of the urine sample was tested per plate. In one set of experiments the urine samples were passed through the XAD-2 column without prior treatment with B-glucuronidase and in the second set of experiments the urine was pretreated with B-glucuronidase (the urine sample adjusted to pH 7.0 was incubated with B-glucuronidase 600 units/ml urine for 18 hours to hydrolyze the sugar conjugates) prior to passing through the XAD-2 column.

The results showed differences in the mutagenic activity of the urine samples from smokers and nonsmokers. The two nonsmokers did not yield values significantly higher than their respective controls. In the absence of B-glucuronidase treatment and microsomal activation, the three smokers also did not show activity much higher than the respective controls. However, after B-glucuronidase treatment one smoker showed somewhat higher mutagenic activity in the absence of the microsome preparation. In the presence of the microsome preparation the urine samples from all three smokers yielded higher activity with or without B-glucuronidase treatment. In two instances B-glucuronidase treatment had little or no influence on mutagenic activity.

The results may be summarized as follows:

1. In the absence of the microsome preparation none of the samples yielded values significantly higher than the controls under the different test conditions.

2. In the presence of the microsome preparation the nonsmokers did not show positive mutagenic activity under the different test conditions.

3. Among smokers, in general, the unhydrolyzed urine showed higher mutagenic activity followed by B-glucuronidase hydrolyzed urine. Acid hydrolyzed and B-glucuronidase plus sulfatase hydrolyzed urine samples showed consistently lower activity. In the case of smokers, acid hydrolysis, as well as sulfatase treatment, resulted in toxic effects (i.e., decreased mutagenic activity). Based on the foregoing, it would appear that it is advisable to use unhydrolyzed urine in the XAD-2 method.

On the basis of these preliminary results, it would appear that the XAD-2 absorption would be the method of choice for analyzing the human urine samples. The XAD-2 absorption method (modified by substituting a 3 cm^3 bed volume of XAD-2 for the 1.5 cm^3 bed volume originally used) was tested on urine samples from Chicago steel workers (smokers and nonsmokers) and from organic farmers (nonsmokers). The results from the organic farmers were all negative. All positive results were found with the steel workers who smoked, although not all smokers yielded positive results. These preliminary results indicate that the XAD-2 absorption method is capable of detecting exposure to mutagenic material in human urine associated with cigarette smoking. However, this is based on a small sample and does not reflect the extent of individual variation in response.

Test data have shown a non-linear response of the Salmonella system to most mutagens. This makes it difficult to determine from dose-response curves how the mutagenic activity of different samples compare. Therefore, a procedure was employed to provide a value for the "relative mutagenic activity" (RMA) of a sample, based on the comparison of 50 known organic noncarcinogens and 50 organic compounds that had been previously shown to be carcinogenic toward laboratory animals. In this comparison a "mutagenic activity ratio" was computed from the quotient $(E - C)/C_{Av}$, where E is the number of mutant colonies obtained from the experimental sample; C is the control value (i.e., the number of mutant colonies observed when the experimental material is not included) obtained on the day of analysis; and C_{Av} is the "historical" control value, or the average control value for all runs carried out during the course of the study. Studies have shown that in the presence of liver microsomes 93% of the non-carcinogens yield mutagenic activity ratios of 2.0 or less and 83% of the carcinogens yield ratios greater than 2.0. About 98% of the noncarcinogens yield ratios of 2.0 or less if microsomes are absent. In reporting analyses, a sample is regarding as possessing statistically significant mutagenic activity if at some concentration it yields a mutagenic activity ratio greater than 2.0. This type of analysis was conducted on air samples from Los Angeles and Pittsburgh.

Several of the air samples were devoid of mutagenic activity toward bacteria strains TA 1538 and strain TA 100. These samples were primarily aliphatic fractions, MAP (Middle A.P. [heavy]), CD (composite dust sample, Allegheny County) and the top stage (20-3.5μ) of the atmospheric sample (AP). There was considerable variation in the relative mutagenic activities of different sample fractions. The highest level of mutagenic activity occurred in aromatic fraction II. Oxy-neutral fractions II and III and aromatic fraction I were somewhat less active, while oxy-neutral fraction I had a low activity and the aliphatic fraction had zero activity. The Pittsburgh acid fraction (PHS-2) and organic extracts were very active. The relative mutagenic activity of the air particulates increased significantly with decreasing particle size, as shown both in Pittsburgh and Los Angeles.

Results were obtained with two different Salmonella strains: TA 1538, which is particularly responsive to frame-shift mutations, and TA 100, which is particularly responsive to base-pair mutations and to certain types of frame-shift mutations. With two exceptions, the relative mutagenic activities of different samples are roughly parallel in the two strains. The aromatic fraction II samples from Pittsburgh showed a disproportionally high effect with TA 100 (in the presence of the microsome preparations), suggesting that it contains a relatively high concentration of base-pair mutations. The Pittsburgh acid fraction (PHS-2) was only slightly active toward TA 100, suggesting that base-pair mutagens are absent, and that the activity was due to frame-shift mutagens which affect TA 1538, but not TA 100.

In some samples, inherently active mutagens (i.e., those which do not require microsomal activation) were apparently present. Where such mutagens occur, the interpretation of the mutagenic activity of the sample when the microsomal preparation is present must remain qualitative. This is based on the fact that some inherently active mutagens are inactivated by the microsomal preparation, while others are not affected. Thus, the mutagenesis test responds to three general classes of mutagens: (a) inherently active mutagens which are not inactivated by the microsome preparations; (b) inherently active mutagens which are inactivated by the microsome preparation; (c) mutagens which are activated by the microsome preparation. Tests conducted in the absence of the microsome preparation measure the activity due to mutagens of classes (a) and (b). Tests conducted in the presence of the microsome preparation are indicative of activity due to mutagens of class (c) plus the activity observed in the absence of the microsome preparation, less the activity of mutagens of class (b). Thus, the value observed in the presence of the microsome preparation is a measure of the mutagens of class (c), only if there is no appreciable activity when the microsome preparation is absent. These constraints are particularly applicable to the values obtained from Pittsburgh organic extract samples, and the atmospheric particulate samples.

The relative mutagenic activities computed from the Los Angeles air particulate samples generally showed an inverse relationship between particle size and mutagenic activity. The relationship between the mutagenic activities of samples collected upwind and downwind from the source are less consistent, although more often than not the activity of the downwind sample is somewhat higher than the comparable upwind one.

Mutagenesis tests on six synthetic fuel oil samples were conducted. Duplicate plates were prepared with successive amounts of the sample dissolved in DMSO. The samples were tested with five different strains of Salmonella, both in the presence and absence of microsomes. None of the samples showed activity toward strains TA 1353, TA 1537, or TA 100. None of the samples showed significant mutagenic activity in the absence of microsomes. A light, hydro-treated oil sample failed to show a positive mutagenic response against any strain. In the concentration range of 100 to 1000 µg, samples of heavy oil, heavy, hydrotreated oil, and filtered, raw pyrolysis oil showed positive mutagenic responses toward strains TA 1538 and TA 98 in the presence of microsomes. The "syncrude" samples showed a weak positive response against the same strains at 1000 µg. The results of the mutagenesis tests of a sample of MADE #4 (fuel oil) showed that in the presence of microsomes the sample was mutagenic toward TA 1538 and TA 1537. Activity toward TA 98 was borderline. In the absence of microsomes the sample showed positive response toward TA 1538 at 1 µg and toward TA 1537 at 50,000 µg. The sample failed to show any mutagenic activity toward strains TA 1538 or TA 100. Based on thin layer chromatography (TLC) analysis this sample contained at least two mutagenically active components, one active toward TA 1538 and the other toward TA 1537.

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Related Research

There is a wide range of related research on in vitro screening techniques both within the Interagency Program and other research agencies and institutions.

B. TASK TITLE:

Determination of the Influence of Materials Concerned with the Extraction of Ores

HERL/RTP TASK NO: 8152

CONTRACTOR: Northrop Services, Inc.

CONTRACT NO: 68-02-2566

Summary

Commercial asbestos of various types are known to cause human disease, notably pulmonary fibrosis, pleural plaque and chronic fibrosis, lung cancer and malignant tumors of the pleura and peritoneum, and possibly cancers in the gastrointestinal tract. These occur in asbestos miners, manufacturers of asbestos products, and workman utilizing such products. Additionally, a number of persons in peripheral contact with mining or mine workers have developed malignancies.

Asbestos is a fibrous silicate which occurs in a number of varieties. Other forms of silicates which occur abundantly in the earth's crust have varying resemblance (in the formation of either fiber or long narrow crystals) to commercial asbestos. Such material is being constantly turned up in various forms by mining, quarrying, road building, etc., it is of great importance to know whether persons exposed to such material are at risk in a similar manner to those exposed to asbestos.

The importance of this sort of information to EPA is attested to by the large number of inquiries, litigations, and the like with which the Agency has to deal when such materials are reported. For the above reasons a research project on the potential toxicity of the non-asbestos fibrous minerals was in October, 1975, when building of a facility was begun. Concomitant with this, a provisional protocol was circulated to informed individuals for comment. These comments were reviewed by an ad hoc study section and a formal protocol was approved in June, 1976. The preliminary biological experimentation began in July, 1976 and intrapleural and intratracheal studies were begun.

Scope and Objectives

This study comprises the following tasks:

1. Intratracheal incoulation of 2000 rats.

2. Intrapleural incoulation of 450 rats.

3. Toxicity in tracheal transplants.

4. *In vitro* studies

   a. RBC lysis tests
   b. Macrophage lysis tests
   c. Toxicity to human fibroblasts W138
   d. Toxicity to Chinese hamster ovary cells (CHO)

5. Retention of fibrous minerals in lung.

6. Relation of fibrous minerals in lung.

7. Influence of mineral fibers on biology of pulmonary macrophages.

8. Interaction of mineral fibers and organic carcinogens.

The objectives are (1) the determination of toxicity of mineral fibers in the elicitation of chronic disease, cancer of the lung, mesatheliomas, and related diseases as related to their mineralogical characteristics and compared to the variations with the asbestos similar parameters, and (2) estimation of health risk of exposure to such fibers during mining, quarrying, and related operations.

Background and Approach

There is a growing body of evidence suggesting the health effects associated with exposure to asbestos may not be unique to the commercial forms, but may also be associated with asbestos-like fibers found in other rocks, and even man-made fibers. It is now known that the external gross appearance of the mineral may not be the determinant but rather its microfibrillar nature which is expressed after crushing or grinding. The toxicity potential of the silicate mineral may, therefore, be related to its proclivity to form micro-crystals of a certain configuration during crushing and grinding. Thus, it is likely that a mode of toxicity, hitherto thought to be associated only with commercial asbestos, may be present at a greater or lesser degree in other fibrous amphiboles.

The approach consists of a closely integrated mineralogical-biological project which compares minerals from various sites containing amphiboles of differing positions on the cummingtonite-grunerite scale, and other minerals. These are selected on the basis of thorough geological study and a certain number are designated for possible biological experimentation. The samples are then prepared uniformly in a manner to assure release of a maximal number of fine crystals, or fibers. Biological experimentation with such material would make possible (1) selection of the worst possible case on the basis of geological prediction, (2) determination of the biological activity on the basis of the specific geological type, and (3) assurance of a concentration of the specific geological characteristics in any given biological experiment. The latter approach maximizes the possibility of determining significant biological activity and relating that activity by dose-response to the type of amphibole rock. This information would be of value not only in a specific locale, but would have wide application in rock operations generally.

The biological study relates the presence or absence of lung cancer, fibrosis in the lung, and mesotheliomas to mineralogical types, using commercial asbestos as a positive control and the injection vehicle (gel saline) as a negative control. The approach in the *in vitro* systems compares lysis and other parameters on the basis of presence or absence of fibers, internal molecular structures, etc.

Research Accomplished

Two thousand animals have been injected with fibrous minerals by intratracheal instillation and intrapleural injection. The highest internal dose was determined, and pulmonary pathological alteration was determined by short-term studies. Comparative retention of amphibole fibers and amosite asbestos fibers determined.

In vitro studies have demonstrated differences in lysis activity between various closely related minerals, including fibrous and non-fibrous varieties. Mineral properties such as surface area, surface charge, and elemental analysis were correlated with the lytic action on mammalian erythrocytes. The same particles were tested for toxicity in rabbit alveolar macrophage systems and Chinese hamster ovary cell systems. Toxicity in these systems seemed to correlate with lysis in mammalian erythrocyte systems.

A report of this work was published in procedings of the Asbestos Workshop sponsored by the National Bureau of Standards. Additional scientific papers are in preparation. An annual progress report was submitted to EPA by the contractor.

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Related Research

Preliminary studies are being performed on rock dusk from oil shale mining and retort particles in interaction with asbestos-like minerals.

C. TASK TITLE:

Study of Metabolic and Physiologic Measurements in Populations with Long-Term Exposure to Pollution from Coal Combustion

HERL/RTP TASK NO: 8166

GRANTEE: University of Akron

GRANT NO: R804256

Summary

This study involves an investigation of the physiologic changes associated with long-term exposure to combustion products of coal with high sulfur content, being carried out in Cleveland, Ohio and Elyria, Ohio. The participants in this phase of the study are from 10 through 60 years old. The health indices compared are:

-accumulation of trace metals in human systems
-pulmonary functions
-cardiovascular function, and
-incidence of hypertension.

Also, current and historic air pollution levels are measured and/or estimated for each of the two communities.

This study also compares frequency and severity of acute respiratory illness in 5th and 6th grade children relative to levels of air pollutants. This component is being carried out in Akron, Ohio. The individual school room teachers are keeping daily diaries of each child's symptoms. Base-line pulmonary function was established for each child at the beginning of the study and the test repeated at six to eight week intervals throughout the school year. After each incidence of acute respiratory illness (ARI), pulmonary function measurements were made daily for two weeks to establish a normal recovery rate. Procedures were developed to retest the pulmonary function of each child subsequent to an air pollution episode. These arrangements will continue in effect during the 1978-1979 school year. No air pollution episodes occurred during the 1977-1978 school year.

Scope and Objectives

The scope of this work falls in two categories:

1. What are the long term physiological effects of exposure to combustion products of coal with high sulfur content? This study involves studying a cross section of population including ages from 10 - 60 years.

2. What are the effects of air pollutants on the incidence and severity of acute respiratory illness (ARI) in grade school children?

Objectives of the long-term exposure (Cleveland vs. Elyria) study are:

1. To determine the levels of accumulation of trace metals in human systems.

2. To determine the reactions of hemoglobin (COHb, MetHb).

3. To assess pulmonary function.

4. To assess cardiovascular function.

5. To determine air quality.

6. To determine if there is a difference in the incidence of hypertension between the two study areas.

Objectives of the acute respiratory illness (Akron) study are:

1. To determine the frequency of ARI in 5th and 6th grade children relative to levels of air pollutants.

2. To determine the severity of ARI in 5th and 6th grade children relative to levels of air pollutants.

3. To examine specific symptoms and possible correlations with air pollutants.

4. To assess the recovery from an ARI relative to the return to normal of certain respiratory volumes and capacities.

5. To determine air quality.

Background and Approach

Long-term Exposure Study--

Air pollution is a problem that is confronting urban populations of the world with increasing regularity. One of the problems that is particularly disconcerting concerning air pollution is its possible effect on the respiratory system. Initial work in this area was conducted by Rosenbaum (1961) who showed that army recruits from urban areas suffered more respiratory infection while in the army than those coming from rural areas. Since the work by Rosenbaum, a number of other authors have supported similar observations in the United States (Lebowitz 1974, Ferris 1970, Shy et al 1970, Shy et al 1973) England (Lawther et al 1974, Holland et al 1969), Japan (Toyahama, Nagahama et al 1969, Watanabe et al 1969), and Canada (Lefcoe 1974).

In 1970, a large population of junior high school students was studied in Barberton and Revere, Ohio. Barberton is an industrial community, while Revere is rural. The results of this study indicated that lung functions such as vital capacity (VC) and forced expiratory volume (FEV_t) were all significantly lower ($p < .01$) in the Barberton group than the Revere group (Mostardi and Martell 1975)

To further investigate this population it was decided to revaluate a a smaller sample of the original population for VC, FEV_t, maximal midexpiratory flow (MMF), and maximal oxygen consumption (VO_2) by the indirect method. This last parameter is a measure of an individual's aerobic capacity which involves a number of physiological factors among which are: oxygen diffusion capacity, cardiac efficiency, and the efficiency of cellular O_2 uptake and metabolism. Because diffusion capacity of oxygen is a function of alveolar vasculature it was decided that this parameter could provide additional information concerning the effects of air pollution on lung parenchymal tissue.

The results of this second study were quite similar to the first. The Barberton group had a mean VC which was lower than the mean of the Revere group ($p < .01$). Neither FEV_t, nor MMF were significantly different, but VO_2max was ($p < .01$). Values of air pollutants in Barberton, although somewhat lower than three years ago, are still considerably higher than in Revere. It was concluded that such pollutants should be considered important contributory factors on the impairment of cardiopulmonary parameters (Mostardi and Loenard 1974).

The following study is designed to expand our earlier work by increasing the age groups, geographic locations and cardiopulmonary parameters studied, and to employ them in a cross-sectional/longitudinal investigation. The comparative effects of air pollutants between a number of age groups, under different pollution conditions, over an extended period of time, has never been attempted, and these kinds of data should provide detailed, qualitative cause and effect relationships between air pollutants and lung function.

Initially three different age groups 10-12, 25-25, and 45-65 are being compared between two different geographic locations of varying levels of air pollution for the cross-sectional aspect, and each of the age groups within a location will be retested for five years, providing longitudinal data. It is believed that within this experimental design the progressive effects of pollutants can be assessed. In this respect these areas have had near constant levels of pollutants for 20 or 30 years. Providing that the older subjects have fulfilled a prerequisite of longevity within a given area, regression equations can be applied to the different age groups within a given location and an extrapolation can be made from age group to age group. These regressed data can then be compared between locations, providing indications of long term trends. As a result, if certain levels of pollutants are determinants of reduced lung function and pulmonary disorders, then by using correlative statistics, an appropriate cause and effect relationship can be made.

Location Selection--

The state of Ohio is a very suitable area for a study designed to assess the effects of air pollutants. In the northern and eastern parts of the state are a number of cities in which the air quality is consistently above the ambient air quality standards, and about 50% of the time is over the once per year levels. There are other cities where the air quality is consistently between the primary standard and the once per year level, and there are a few areas where the levels are consistently below the primary standard. As a result, with a minimum of travel time large pools of subjects are available who have been exposed to these ambient conditions for nearly a lifetime.

The eastern portion of Cleveland, Ohio has been chosen as the area of high pollution. There is a large industrial complex west of our study zone which includes chemical plants, steel mills, and coke ovens. This area constitutes one of the heaviest polluted areas in the U.S. The clean urban area has not yet been selected, but we currently are monitoring air quality in Elyria for possible use as a site.

Subject Selection--

It is anticipated that approximately 125 - 150 individuals in each age group will be interviewed for their availability and willingness to participate in this study. Age groups are as follows:

1. 10 - 12
2. 25 - 45
3. 45 - 65

The variables to be measured include the following:

1. Vital data - derived from adult questionnaires (see Appendix 1)
2. Pulmonary functions testing (PFT) including vital capacity (VC), forced expiratory volume (FEV_t), and maximal mid-expiratory flow (MMF).
3. Maximal expiratory flow volume (MEFV) curves while breathing room air and also while breathing helium-oxygen ($He-O_2$) 80%-20%.

There is a considerable amount of information in the literature which suggests that MEFV curves using both air and He-O_2 can provide more information concerning the nature of the small airways than FEV_t of MMF (Antic and Macklem 1976, Chan-Yeung at al 1976, and Dosman et al 1975). For this reason we have established MEFV curves as an important aspect of this study and because it is somewhat new to this research design, we feel that it deserves further explanation.

A schematic drawing of the setup is shown in Figure 1. Data collection involves the following steps. The subject is seated comfortably so that his/her mouth is directly in from of a Collins Triple J valve with attached mouthpiece. The subject is instructed as to how a forced expiratroy maneuver is performed, accompanied by a demonstration by the experimenter. Then, attached to the mouthpiece and with a nose clip in place, the subject attempts several trial maneuvers. When the experimenter is satisfied that the maneuver is being performed adequately, the expired air is directed into the dry rolling seal spirometer by opening stopcock 2, and three forced expiration efforts are recorded on the x-y recorder. When three similar curves have been recorded using room air, the same procedure is followed using the He-O_2 mixture.

Prior to breathing the He-O_2 mixture, the subject is taken off the mouthpiece and receives a brief description of the gas mixture, notably that the mixture is somewhat cooler than room air and that the nitrogen normally found in the air has been replaced by another gas. Following the explanation, the subject is reconnected to the mouthpiece with the nose clip attached and then after turning stopcock 1, the subject breathes the He-O_2 mixture out of the 200 liter Douglas bag. To displace the N_2 in the lungs, the subject performs three VC maneuvers with several tidal breaths in between. Following equilibration with the He-O_2 mixture the subject performs forced expiratory maneuvers until three satisfactory maneuvers have been recorded.

The forced expiratory recording are analyzed according to the previously described method of Antic and Macklem (1976). The analysis involves superimposing the curves for air and He-O_2 at total lung volume (TLC). The best FVC for air is used to define the volumes at which $\dot{V}_{max}$ is measured. Thus, the 50% and 75% expired FVC volumes from the best air FVC is used to determine air $\dot{V}_{max50\%}$ and $\dot{V}_{max75\%}$ volumes from all curves. The values $\dot{V}_{max50\%}$ and $\dot{V}_{max75\%}$ are calculated as the difference between the air and He-O_2 curves and expressed as a percentage of the total volume on air at the point. The $V_{iso\dot{V}}$ point is the point at which there is clear crossing or merging of the air and He-O_2 curves as opposed to an apparent converging.

4. Low level exercise tests - Electrocardiographic recordings are carried out at rest, during exercise on a bicycle ergometer, immediately at recovery, and 3 minutes post exercise. The strips are coded using the Minnesota Method.

5. Blood withdrawal - Blood is withdrawn from an antecubital vein and aliquots are used for the following tests:

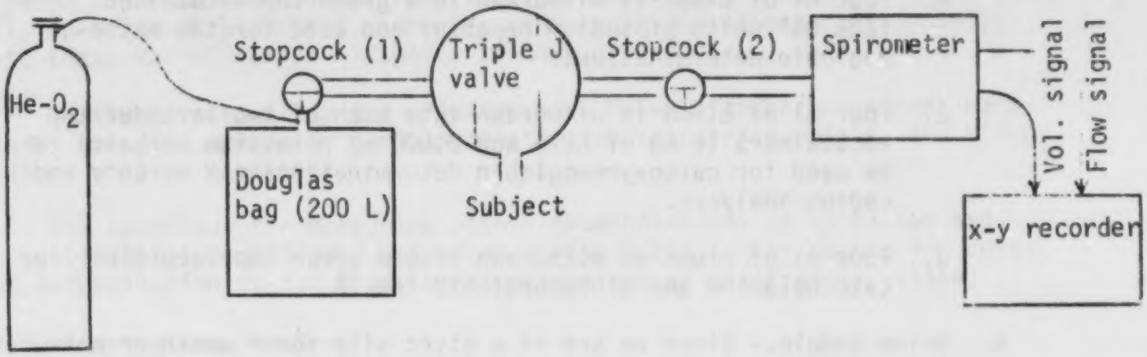
He-O_2
Stopcock (1)
Douglas bag (200 L)
Triple J valve
Subject
Stopcock (2)
Spirometer
Vol. signal
Flow signal
x-y recorder

Figure 1. Schematic for recording MEFV curves

a. Seven ml of blood is withdrawn in a red top Vacutainer (no preservative). The sample is centrifuged at 2500 rpm and the serum removed for alpha-1-anitrypsin, total protein, and immunoelectrophoresis determinations.

b. Four ml of blood is withdrawn in a green top Vacutainer (286 USP units of sodium heparin) and used for the methemoglobin determinations.

c. Four ml of blood is withdrawn into each of two lavender top vacutainers (6 mg of EDTA and 0.008 mg potassium sorbate) to be used for carboxyhemoglobin determinations and mercury and cadium analyses.

d. Four ml of blood is withdrawn into a green top Vacutainer for catecholamine and glucocorticoid levels.

6. Urine sample - Since we are at a given site for a month or more, it is relatively simple to get a 24 hour urine sample. For this purpose we provide 4 liter wide mouth plastic jugs containing 5 ml 0.1 $NHNO_3$ as a preservative, which are given to the subject in the morning and returned the following morning. After measurment of total urine volume, an aliquot ofthe urine is placed into a clean 10 ml plastic vial. This sample is sent to the Trace Metals Laboratory at Metropolitan General Hospital where it is analyzed for mercury and arsenic.

All of the data are entered into an appropriate computer file for statistical analysis.

Acute Respiratory Illness Study--

The effects of Acute Respiratory Illness (ARI) on pulmonary functions in school children have not been studied extensively, nor has the degree to which lung function returns to normal been assessed.

There is also a paucity of data relating air pollution to the incidence and severity of ARI's in children. The study conducted by Collier et al. (1978), in which pulmonary functions were evaluated during and one month after upper respiratory infection, did not include correlation with air pollution. In the study by Levy et al.(1977) the number of hospital admissions for acute respiratory disease in children was positively correlated ($p < 0.01$) with air pollution indices.

The purposes of this particular aspect of the study are (1) to determine if a decrease in pulmonary function is related to incidence and severity of ARI's in school children, and (2) to determine if air pollution indices are correlated with incidence, severity, and convelescent time.

Hypertension Study--

Evidence is accumulating which suggests that stress and social change can influence blood pressure (Bronson and Eleftheriou 1965, Ely et al.1974, Henry et al. 1975, Henry et al. 1967, Henry et al. 1971, Nestel 1969, Nestel and Doyle 1968, Nuckolls et al. 1972). Also there is evidence that coping ability can influence specific neuroendocrine response patterns (Conner et al. 1971, Jones et al. 1970, Kvetnansky and Mikulaj 1970, Kvetnansky et al. 1970, Mason 1968, and Mason 1968b). Finally there is evidence that specific behavior patterns can influence blood pressure and neuroendocrine response patterns (Bronson and Eleftheriou 1965, Christian et al. 1965, Henry et al. 1971, Nuckolls et al. 1972). However, the cause and effect relationships among these variables are virtually unknown.

In the present study the relationships between three behavioral parameters, specific neuroendocrine correlates, certain trace metals, and blood pressure will be examined.

The technique for measuring plasma catecholamines is by Passon and Peuler (1973) as modified by Upjohn Co., (Cat-A-kit). The method for cortison determination is the fluorimetric technique of Glick et al. (1964).

Air Monitoring Component--

In support of the study of air pollution on physiological parameters, seven air monitoring stations have been established in Cleveland, Akron, and Elyria. The stations are located in the section of each city where the subjects for the study live, and will provide an indication of the quality of air in the region, as well as variations in air quality over relatively short distances. The three areas were selected, based on available data which indicated that Cleveland was a region of very high pollution, Akron moderate, and Elyria relatively clean air.

At each of the sites 24 hour samples of total suspended particulates (TSP), sulfur dioxide (SO_2), and nitrogen dioxide (NO_2) are being obtained. The sites are a part of the "CHAMP" system and all analyses are being made by the "CHAMP" contractors. In addition to the TSP, SO_2, and NO_2 measurements, the filters with the total suspended particulates are being analyzed for sulfates, nitrates, polynuclear aromatics, benzene solubles, lead, berryllium, arsenic, cadmium, selenium and mercury.

In addition to the data acquired from the above stations, the TSP, SO_2, and NO_2 measurements from stations in the area operated by the local Air Pollution Control Agency are being obtained and will be used to estimate the air pollution gradient for the region.

The major pollutants in southeast Cleveland come primarily from the industrial area, which includes all levels of steel manufacturing, coke manufacturing, heavy inorganic chemicals, electrical generating stations and transportation sources. The area selected for the study is located just east of the steel and coke manufacturing facilities and based upon the Cleveland Air Pollution data has the highest levels of Air Pollution TSP, SO_2, and NO_2 in the Cuyahoga County and in the state of Ohio.

The major pollutants in Akron come primarily from the production of industrial steam for the rubber industry, electrical power generation, and transportation. The industrial steam is generated using either coal, gas or oil with most coming from oil and gas. The rubber plants have other odor causing organic emissions which are not being analyzed during this project.

Elyria is a rather small community with light industry and there are no large heavy industrial sources of pollutants in the community. The Cleveland Electric Illuminating Company has a large electric generating station at Avon Lake which is about 20 miles northeast of the chosen sites and U. S. Steel has a major installation in Lorain which is about 10 miles north of the area being used in the study. The U. S. Steel Plant has made extensive use of wet scrubbers and is one of the better controlled steel plants in the country. With the prevailing winds coming from the southwest and west, these two plants should have a minimal effect on the air quality in the region. The areas to the south and west of Elyria are agricultural with the closest major industrial complex being at least 60 miles south.

As part of the study, the differences which are found in the levels of the above pollutants for the three areas will be used along with the physiological data to determine if there are correlations between specific pollutants and physiological effects. In addition, the pollution gradients in the areas will be studied to see how the pollution varies from one location in the area to another.

The data will be analyzed to determine if there are significant differences in not only the levels of pollutions, but the ratio of the various pollutants. This information along with weather information obtained from The U. S. Weather Bureau will be used to determine if correlations of air quality, the type of pollutants, and ratios of various pollutants can be related to the wind direction and speed. Studies from New York City (Goldstein and Landovitz 1977a, Goldstein and Landovitz 1977b) indicated that this is not feasible, but because of the location, type of terrain and differences in types of buildings, the New York conclusions are probably not applicable to this study.

Studies in St. Louis (Pooler 1966) indicate that the modeling techniques using a modified Gaussian procedure seem to be applicable for predicting the pollutant levels if the proper choice of dispersion parameters is made. Studies by Yen (1971) indicate that these procedures are better for validating long term averages than daily or weekly changes. Therefore an attempt will be made to relate the pollution levels to the climatic conditions, including wind direction, velocity, precipitation, insulation, season and period of the week or month.

Research Accomplished

Long-Term Exposure Study--

At this date, March 28, 1978, we have tested 250 adults in east Cleveland, Ohio. It takes about 30 minutes to complete all of the tests, and we normally complete 10-12 per day. Most of our subjects have been in the 45-65 age group and we are pursuing locations where we can test younger people. Unfortunately, at this time we do not have any subjects in the 10-12 age group. This is due to the fact that the City of Cleveland School System has refused to cooperate with us and will not allow us to seek volunteers in the grade schools of the area. We did receive some help from the Catholic Dioceses of Cleveland but the enrollment of the one school in the immediate area is small and when we sent our information package home, we received a poor return.

The data that we have collected has been processed and most of it is on disk storage at the main university computer.

Animal Model Studies--

In December of 1977, five pregnant Balb/c females from the Kirchbalm Memorial Mouse Colony were placed in one of our research sites in the East Cleveland area. Within one week all five of the females bore litters, with the total number of mice being 26. At the same time, a similar sample size was established in Akron, Ohio and served as the control group.

After three months all of the experimental mice and 12 of the control mice were sacrificed. Blood was collected for mercury and cadium determinations and hair was shaved from the back for lead, mercury, beryllium, arsenic, selenium and cadmium determinations. The thorax was opened and the lungs and liver were removed and weighed. Tissue samples from each lung lobe and selected liver samples were removed and fixed at 10% buffered formalin for histologic examination. All of these tissue samples are currently being processed.

Acute Respiratory Illness Study--

Two Akron Public City Schools (situated 2 miles apart) were chosen for this study on the basis of their proximity to the rubber factories. Questionnaires (Appendix 2) were sent home to all 5th and 6th grade students. Those returning completed questionnaires which included hair samples for heavy metal analyses comprised the group for our study. All subjects were thoroughly coached in pulmonary function maneuvers. Spirometry was performed with a Warren E. Collins 9 liter water-filled spirometer, measuring Vital Capacity (VC), Forced Expiratory Volume (FEV), and Mid-Maximal Flow (MMF). These measurements were all done in reproducible triplicate (greater than 5% difference rated unacceptable). Prior to each spirometry maneuver, the height and weight of the subject were also recorded.

Pulmonary Function baselines were performed on all subjects after first determining that they were asymptomatic. Thrice weekly, the research team checked for ARI incidents among the subjects. This was done with the aid of both personal interviews and symptom charts which each volunteer was asked to complete daily (See Appendix 3). Pulmonary function measurements were then taken on subjects who indicated the presence of an ARI. During this symptomatic phase of the ARI, spirometry was performed twice on the subject, two days apart. At this time each of the subject's symptoms was graded for relative severity, and labeled light, moderate, or severe.

Careful observation was maintained on the subjects with ARI's and when they were asymptomatic, spirometry was performed three times. These asymptomatic tests were carried out over a nine day period and the purpose was to assess the nature and degree of convalescence.

Hypertension Study--

Presently, (March 28, 1978) we have accumulated hypertension data on 150 subjects in the Cleveland study area. These data are currently being programmed into our computer center for detailed analysis. Statistical tests are being performed comparing blood pressure to heavy metal levels in blood, urine, and hair samples, life style index, plasma catecholamines, and gluccocorticoids.

Air Monitoring Study--

The Akron stations have been run since October, the Cleveland stations since November and the Elyria stations since December and as yet no data have been received from the "CHAMP" contractors. It is imperative that this data be received as soon as possible to enable the data analysis and comparison work for the project to begin.

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Appendix 1. Adult Questionnaire

University of Akron Research Study

On Respiratory Disease

ADULT QUESTIONNAIRE

Please answer the questions in this questionnaire as completely as possible. The questions can be answered by circling the number of the best answer or by filling in a blank with a number or word.

Example: What is your sex:
1. male
[✓ 2] female

If you have any problems in answering any of the questions, a member of the staff will be happy to help you.

ALL INFORMATION WILL BE KEPT CONFIDENTIAL

Name ________________________________ QID ________________

Address ____

Phone ________________________

Social Security No ___-___-___

Date of Birth ________________

Appendix 1. (Continued)

1. What is your full name? ______________________________

2. Date questionnaire completed? ____________ (month) ________ (day) 19______ (year)

3. What sex are you?
   1. Male
   2. Female

4. What is your ethnic group or ancestry?
   1. White
   2. Mexican-American
   3. Black
   4. Indian
   5. Oriental
   6. Other

5. What is your marital status?
   1. Married
   2. Separated
   3. Never Married
   4. Widowed
   5. Divorced

6. How many years of formal education or schooling have you had? (For example, completion of high school = 12) ______________ (years)

7. What is your birthdate? _____/_______/_______ mo. day year

8. How long have you lived in your present city or town?
   1. 1-5 years
   2. 5-10 years
   3. 10 or more years

9. How long have you lived at your present address?
   1. 1-5 years
   2. 5-10 years
   3. 10 or more years

10. Do you anticipate moving from your present address in the next year?
    1. Yes
    2. No

11. Do you usually cough first thing in the morning in bad weather? (If you usually cough in the morning regardless of the weather, circle Yes.
    1. Yes
    2. No

12. Do you usually cough other than in the morning in bad weather? (If you usually cough regardless of the weather, circle Yes.)
    1. Yes
    2. No

If you answer is Yes to either questions 11 or 12, proceed to a:

a. Do you cough on most days for as much as three months of the year?
   1. Yes
   2. No

Appendix 1. (Continued)

a.	For how many years have you had this cough?	1. Less than 2 years 2. 2-5 years 3. More than 5 years
b.	Do you cough more or less now than you did two years ago?	1. More 2. Less 3. No Change
d.	During the past two years, have you seen a doctor about your cough?	1. Yes 2. No
13.	Do you usually bring up phlegm, sputum, or mucous from your chest first thing in the morning in the bad weather? (If you usually bring up phlegm, sputum, or mucous from you chest in the morning regardless of the weather, circle Yes)	1. Yes 2. No
14.	Do you usually bring up phlegm, sputum, or mucous from your chest at other times during the day or night in bad weather? (If you usually bring up phlegm, sputum, or mucous from you chest, regardless of the weather, circle Yes)	1. Yes 2. No

If your answer is Yes to either question 13 or 14 proceed to a:

a.	Do you bring up phlegm, sputum, or mucous from your chest on most days for as much as three months of the year?	1. Yes 2. No
b.	For how many years have you raised phlegm, sputum, or mucous from your chest?	1. Less than 2 years 2. 2-5 years 3. More than 5 years
c.	Do you raise more or less phlegm, sputum, or mucous from your chest now than you did two years ago?	1. More 2. Less 3. No change
d.	During the past two years, have you seen a doctor about this condition?	1. Yes 2. No
15.	Does your chest sound wheezy or whistling?	1. Yes 2. No

If your answer is Yes, proceed to 15a:

a.	Do you get this with colds?	1. Yes 2. No

Appendix 1. (Continued)

b.	Do you get this even when you don't have a cold?	1. Yes 2. No
c.	Do you get this on most days?	1. Yes 2. No
d.	During the past year, has your wheezing or whistling improved, worsened, or stayed the same?	1. Improved 2. Worsened 3. Stayed the same
e.	How old were you when you first started wheezing?	________(age)
16.	Have you ever had attacks of shortness of breath with wheezing?	1. Yes 2. No
	if your answer is Yes, proceed to 16a	
a.	Do you get this with colds?	1. Yes 2. No
b.	Do you get this even when you don't have a cold?	1. Yes 2. No
c.	During the past year, how many attacks of shortness of breath with wheezing did you have?	1. None 2. A few (1-3) 3. Several (4-10) 4. Many (13 or more) 5. Almost every day
d.	How old were you when you had your first such attack?	________(age)
e.	How old were you when you had your last such attack? (If you still have attacks, indicate your present age)	________(age)
17.	Are you more short of breath than most people your age?	1. Yes 2. No
18.	Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill?	1. Yes 2. No
19.	Do you get short of breath walking with other people of your own age on level ground?	1. Yes 2. No
20.	Do you have to stop for breath while walking at your own pace on level ground?	1. Yes 2. No
21.	Did you have any respiratory trouble before age 16?	1. Yes 2. No

Appendix 1. (Continued)

22.	During the past three years, how much trouble have you had with illnesses such as chest colds, bronchitis, or pnuemonia? (Does not refer to head colds)	1 2 3 4 5 Some a great deal (Circle appropriate number)
23.	During the past three years, how often were you unable to do your usual activites because of illnesses such as chest colds, bronchitis, or pneumonia? (Does not refer to head colds)	1. Never 2. During one such illness 3. During 2-5 such illnesses 4. During 6 illnesses or more
24.	Do you think you have ever had any of these chest disorders: asthma, any kind of bronchial trouble, emphysema?	1. Yes 2. No
25.	Have you ever seen a doctor for asthma?	1. Yes 2. No
	If you answer is Yes, proceed to 25a:	
	a. During the past year, have your symptoms improved, worsened, or stayed the same?	1. Improved 2. Worsened 3. Stayed the same
	b. During the past year, have you had a period as long as two weeks in which you had no breathing problems at all even with exercise?	1. Yes 2. No
	c. How quickly are your attacks usually relieved by treatment?	1. Within minutes 2. Within hours 3. Within days 4. Not relieved 5. No treatment used
26.	Have you ever seen a doctor for chronic bronchitis?	1. Yes 2. No
	If your answer is Yes, proceed to 26a:	
	a. During the past year, have your symptoms improved, worsened, or stayed the same?	1. Improved 2. Worsened 3. Stayed the same
	b. Have you had medication or treatment for it?	1. Yes 2. No

Appendix 1. (Continued)

27. Have you ever had any of the following (If uncertain circle No):

a. Emphysema
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

b. Bronchiectasis
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

c. sinus trouble
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

d. pneumonia or bronchopneumonia
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

e. tuberculosis
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

f. valley fever (coccidiodomycosis)
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

g. Histo (histoplasmosis)
1. Yes, I still have it
2. Yes, but I no longer have it
3. No

h. any other respiratory disease
1. Yes
2. No

If yes, please specify ____

28. How long have you been employed at your current occupation?
1. 1-5 years
2. 5-10 years
3. 10 or more years

29. Have you in the past worked in a dusty job or where there has been irritating gas, chemical fumes, or smoke?
1. Yes
2. No

If your answer is Yes, proceed to 29a:

How many years were your employed there?
1. 1-5 years
2. 5-10 years
3. 10 or more years

Appendix 1. (Continued)

b.	Was the exposure to the irritating gas, chemical fumes, smoke, or dust mild, moderate or severe?	1. Mild 2. Moderate 3. Severe
c.	How many different companies have you worked for in the last 10 years?	_______ (Number)
30.	Do you now smoke cigarettes?	1. Yes 2. No
	If your answer is Yes, proceed to 30a: If your answer is No, proceed to question 31.	
a.	Do you inhale?	1. Yes 2. No
b.	Do you smoke cigarettes with filters?	1. With filters 2. Without filters 3. Both with and without filters
c.	How many cigaretts do you usually smoke each day at the present time? (Please give best estimate: one pack contains 20 cigarettes).	_____number per day
d.	How old were you when you began to smoke cigarettes?	_____ (age)
e.	What is the usual number of cigarettes you have smoked per day since you began to smoke? (Please give best estimate: one pack contains 20 cigarettes)	_____number per day
f.	Are you smoking less now than two years ago?	1. Yes 2. No
g.	If so, how many are you smoking now?	_______number per day
	If you have completed this section, skip question 31 and go to question 32	
31.	If you do not smoke cigarettes now, did you ever smoke them regularly or occasionally?	1. Never 2. Regularly 3. Occasionally
	If your answer is regularly or occasionally, proceed to question 31a:	
a.	What was the usual number of cigarettes you smoked per day? (Please give the best estimate: one pack cigarettes contains 20 cigarettes)	______ number per day
b.	Did you inhale?	1. Yes 2. No

Appendix 1. (Continued)

c. How old were you when you began to smoke cigarettes? ______ (age)

d. How old were you when you stopped smoking cigarettes regularly? ______ (age)

e. Were you influenced to stop because you had a cough, wheezing, or shortness of breath?
1. Yes
2. No

32. Do you now smoke pipes or cigars?
1. Yes
2. No

If your answer is Yes, proceed to 32a:

If your answer is No, proceed to question 33.

a. How many pipefuls or cigars do you usually smoke each day? _____number per day

b. How old were you when you first smoked? _____(age)

c. Do you usually inhale when you smoke either pipes or cigars?
1. Yes
2. No

(If you have completed this section, skip question 33 and go to question 34)

33. If you do not smoke cigars or pipes now, did you ever smoke them regularly or occasionally?
1. Never
2. Regularly
3. Occasionally

If your answer is regularly or occasionally, procced to 33a:

a. How many pipefuls or cigars did you usually smoke each day? _____number per day

b. How old were you when you first smoked pipes or cigars? _____(age

c. How old were you when you stopped smoking pipes or cigars? _____(age)

d. Did you usually inhale when you smoked either pipes or cigars?
1. Yes
2. No

34. Do you ever have paid or discomfort in your chest brought on by exertion or excitment and is relieved by rest?
1. Yes
2. No

Appendix 1. (Continued)

35. Have you ever been told by your doctor that you had angina or angina pectoris?
1. Yes
2. No

36. Do you have to stop to catch your breath while walking up one (1) flight of stairs?
1. Yes
2. No

37. Have you ever been hospitalized for a heart attack?
1. Yes
2. No

38. Are you currently taking any medication?
1. Yes
2. No

39. If Yes to question 38, what is the name of the medication? ____________ ________________

Appendix 2. Pediatric Questionnaire

University of Akron Research Study
on Respiratory Disease

PEDIATRIC QUESTIONNAIRE I

We would like the questionnaire to be completed by the parent of each child participating in the study. The questions can be answered by circling the number of the best answer or by filling in a blank with a number or word. All questions in this questionnaire concern only your child. Please try to answer all questions as completely as possible.

ALL INFORMATION WILL BE KEPT CONFIDENTIAL

Name ______________________________ ID __________

Address ______________________________

City, State, Zip Code __________________ Phone __________

Child's date of birth __________________ Sex __________

Appendix 2. (Continued)

1. Date questionnaire completed? ________ (month) ________ (day) ________ (year)

2. Does this child suffer from shortness of breath while walking?
   1. Yes
   2. No

3. Does this child suffer from shortness of breath while playing?
   1. Yes
   2. No

4. Does this child ever stop to catch his breath while walking?
   1. Yes
   2. No

5. Does this child ever stop to catch his breath while playing?
   1. Yes
   2. No

6. Does this child get short of breath walking with other shildren the same age on level ground?
   1. Yes
   2. No

7. Does this child have to stop for breath while walking at his or her own pace on level ground?
   1. Yes
   2. No

8. Does he or she become short of breath, during normal play, more often now than two years ago?
   1. Yes
   2. No

9. During the past two years, has this child seen a doctor because of shortness of breath?
   1. Yes
   2. No

10. During the past two years, how often was this child unable to do his/or her usual activities because of illnesses such as chest cold, bronchitis, or pneumonia? (Does not refer to head colds.)
    1. Never
    2. During 1 such illness
    3. During 2-5 such illnesses
    4. During 6 or more illnesses

11. During the past year, how many days has this child been unable to do his or her usual activities because of such illnesses? ________(days)

12. During the past year, has this child seen a doctor for: (Consult your physician if you are unfamiliar with these terms.)

    a. Emphysema?
       1. Yes
       2. No

    b. Chronic bronchitis?
       1. Yes
       2. No

Appendix 2. (Continued)

c. Bronchiectasis?
1. Yes
2. No

d. Asthma?
1. Yes
2. No

e. Sinus trouble?
1. Yes
2. No

f. Pneumonia?
1. Yes
2. No

g. Acute bronchitis?
1. Yes
2. No

h. Asthmatic bronchitis?
1. Yes
2. No

i. Bronchiolitis?
1. Yes
2. No

j. Croup
1. Yes
2. No

k. Whooping cough?
1. Yes
2. No

l. Eczema?
1. Yes
2. No

m. Any other respiratory disease?
1. Yes
2. No

If yes, specify ____

13. Has this child ever had hay fever or any other condition that makes the nose runny or stuffy apart from colds?
1. Yes
2. No

If yes to 13, proceed to 13a:

a. How old was this child when this condition was first noticed? _______ (age)

b. How old was this child when he or she last had this condition? (If he or she still has it, indicate present age.) _______ (age)

Appendix 2. (Continued)

c. Has a doctor ever said this condition was due to an allergy?	1. Yes 2. No
14. During the past year, has he or she had an allergic reaction to any food or medicine?	1. Yes 2. No
15. During the past year, has this child received allergy shots? (Does not refer to allergy skin tests.)	1. Yes 2. No
16. Is this child ever troubled by redness, itching, or burning of the eyes?	1. Yes 2. No

If yes to 16, proceed to 16a:

a. Circle the months in which these eye symptoms are most severe.

OR: ______ Check here if no relation to time of year.

1	2	3	4	5	6	7	8	9	10	11	12
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

b. Does he or she have this on most days for at least one month of the year?	1. Yes 2. No
c. Do you think this eye problem is due to an allergy?	1. Yes 2. No
d. Has a doctor ever said it was due to an allergy?	1. Yes 2. No
17. During the past year, has this child had a chest X-ray?	1. Yes 2. No
18. During the past year, has this child been hospitalized for any heart or lung problem?	1. Yes 2. No
19. Has this child ever had any heart or lung surgery?	1. Yes 2. No
20. Where would you like this child's test results from this study sent?	1. Doctor 2. Myself 3. Nowhere

21. What is the doctor's name and location: Name ________________________

Address ________________________ City, State, ZIP ______________________

Appendix 2. Pediatric Questionnaire II (Continued)

University of Akron Research Study
on Respiratory Disease

PEDIATRIC QUESTIONNIARE II

ALL INFORMATION WILL BE KEPT CONFIDENTIAL

Name ______________________________ ID ______________

Address ____

City, State, Zip Code ____________________ Phone __________

Child's date of birth ____________________ Sex __________

Appendix 2. (Continued)

1. Date questionnaire completed? ________ (Month) _____ (Day) ______ (Year)

2. During the past two years, has this child had: (If uncertain, circle No)

   a. any kind of heart trouble?
      1. Yes
      2. No

   b. high blood pressure?
      1. Yes
      2. No

3. Does this child have a cough when he or she doesn't have a cold? (Either first thing in the morning or at any other time of the day.)
   1. Yes
   2. No

   If Yes, to 3, proceed to 3a:

   a. At what time of day does this cough usually occur? (Circle correct answer(s).)
      1. In the morning (shortly after rising)
      2. Later in the morning
      3. Afternoon
      4. Evening
      5. During the night
      6. No relation to time of day

   b. Are there any months in which this child coughs on most days?
      1. Yes
      2. No

      If Yes, please specify the number of months per year. ____________(months)

   c. Circle the months in which the cough is usually most severe; or _____ check here if no relation to time of year.

1	2	3	4	5	6	7	8	9	10	11	12
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

   d. For how many ears has he or she had a cough?
      1. Less than 2 years
      2. 2-5 years
      3. More than 5 years

   e. Does the weather affect the cough?
      1. Yes
      2. No

Appendix 2. (Continued)

f.	Does this child cough more or less now than two years ago?	1. More 2. Less 3. No change
g.	During the past two years, has he or she seen a doctor about the cough?	1. Yes 2. No

4.	Does this child ever bring up phlegm (flem), sputum, mucous, or any type of fluid from the chest when he or she doesn't have a cold? (Either first thing in the morning or at any other time of the day.)	1. Yes 2. No

If Yes to 4, proceed to 4a:

a.	At what time of day does this usually occur? (Circle correct answer(s))	1. In the morning (shortly after rising) 2. Later in the morning 3. Afternoon 4. Evening 5. During the night 6. No relation to time of day
b.	Are there any months in which he or she brings up phlegm, sputum, or mucous from the chest on most days?	1. Yes 2. No
	If Yes, please indicate the number of months per year	________ (months)
c.	Circle the months in which this is usually most severe	

OR ______ Check here if no relation to time of year

1	2	3	4	5	6	7	8	9	10	11	12
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

d.	For how many years has he or she raised phlegm, sputu, or mucous from the chest?	1. Less than 2 years 2. 2-5 years 3. More than 5 years
e.	Does the weather affect this condition?	1. Yes 2. No

Appendix 2. (Continued)

f.	Does this child raise more or less phlegm, sputum, mucous, or any type of fluid from the chest now than two years ago?	1. More 2. Less 3. No change
g.	During the past two years, has he or she seen a doctor about this condition?	1. Yes 2. No
5.	Does this child's chest ever sound wheezy or whistling?	1. Yes 2. No
	If Yes, to 5, proceed to 5a:	
a.	Does he or she get this with colds?	1. Yes 2. No
b.	Does he or she get this even without having a cold?	1. Yes 2. No
c.	Does he or she have a wheezy sound on most days?	1. Yes 2. No
6.	Has this child ever had attacks of shortness of breath with wheezing?	1. Yes 2. No
	If Yes to 6, proceed to 6a:	
a.	Does he or she get this with colds?	1. Yes 2. No
b.	Does he or she get this even without having a cold?	1. Yes 2. No
c.	During the past year, how many attacks or shortness of breath with wheezing did this child have?	1. None 2. A few (1-3) 3. Several (4-10) 4. May (11 or more)
d.	How old was this child when he or she had the first such attack?	___________ (age)
e.	How old was this child when he or she had the last such attack? (If he or she still has attacks, indicate present age.)	___________ (age)
7.	Where would you like this child's test results from this study sent?	1. Doctor 2. Myself 3. Nowhere

Appendix 2. (Continued)

8. What is the doctor's name and location?

____ Name

____ Address

____ City, State, Zip

Appendix 3. Pediatric Symptom Chart

Name ______________ Home Room ______________ Week ______________

	Saturday		Sunday		Monday		Tuesday		Wednesday		Thursday		Friday	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Cough														
Stuffy or Runny Nose														
Sore Throat														
Chest Congestion (Wheezy chest)														
Eye Irritation														
Cold														

D. TASK TITLE:

Implementation of Screening Test for Potentially Hazardous Airborne Particulate Materials Using the Alveolar Macrophage Test System

HERL/RTP TASK NO: 8150

CONTRACTOR: Northrop Services, Inc.

CONTRACT NO: 68-02-2566

Summary

The contractor has implemented the Rabbit Alveolar Macrophage (RAM) Test System with model particulates as well as particulates and other samples from conventional combustion sources and alternative energy sources. Emphasis has been placed on particulate samples due to the macrophages capability to engulf enhaled particulate. A series of toxicity end points have been compared and optimal end points and test conditions selected for routine screening.

Using these techniques a total of 40 environmental samples have been evaluated. Since many of these samples were found to have a low toxicity in the RAM, current research is focusing on comparing potentially more sensitive clonal cytotoxicity assays with the RAM assay using selected particulates.

Scope and Objectives

The objective of this task is to implement the RAM bioassay with model particulate compounds in such a manner than the sensitivity is optimized and test conditions standardized. Subsequently actual environmental samples would be evaluated using this toxicity bioassay system. This approach will allow evaluation not only of a series of environmental samples including conventional and alternative combustion source particulate, but also will permit evaluation of this bioassay system.

Background and Approach

Studies in this and other laboratories have defined a central role for the alveolar macrophage in the defense of the lung against inhaled particulate materials. We have identified a number of metallic substances of the type known to be present in air effluents which are relatively toxic for the alveolar macrophage (Cd, V, Ni, Hg, etc.). Many substances such as some metals and certain organics are emitted in the vapor phase and subsequently preferentially condense on the surface of the smaller respirable particulate materials. We now have a number of chemical analytical techniques and biological end points available to study the nature of the surfaces of particles and the resulting effects of the *in vitro* exposure of these particles to alveolar macrophage. The biological end points form the basis of a valuable screening system for potentially hazardous airborne particulate materials.

In collaboration with analytical chemists who are able to define the surface chemical properties of particulate samples, a systematic screening effort is being carried out to define the biological response of the alveolar macrophage to phagocytized particles *in vitro*. Effort has focused on the propensity of crude particles to promote cell lysis, to affect phagocytic activity (as determined by depression in adenosine triphosphate levels), to alter the integrity of the lysosomes, and to induce enzyme systems capable of activating potentially carcinogenic compounds such as the polynuclear aromatics which may be adsorbed to particulate surfaces. The influence of the nature of the surface of standard particles on the extent of adsorption of materials has been examined.

Research Accomplished

Metal-coated fly ash particles were utilized as model substances in evaluation and implementation of *in vitro* particulate testing with rabbit alveolar macrophages (RAM). Several toxicity end points were measured in the RAM cells following exposure of the cells to the metal-coated particulates in dose response and time sequence studies. Cdo-coated particles have a significant soluble component, whereas the MnO and NiO-coated particles are insoluble. The earliest end point effected in these studies was cellular adenosine triphosphate (ATP) levels. Cellular viability and protein concentrations were also significantly depressed at higher concentrations. These particulate samples were utilized to select optimal test conditions and end points for evaluating the relative toxicity of particulate samples. A standardized protocol was developed.

Forty environmental samples have been evaluated in this bioassay including respirable particulate from:

1. conventional coal combustion
2. fluidized bed combustion
3. coal gasification

None of these particulates were found to be highly toxic. In fact it was generally impossible to determine EC_{50} values due to the low toxicity to the RAM.

Clonal assays with continuous cell lines which are generally more sensitive toxicity test systems have been considered inappropriate for testing particulates. As a part of this research, clonal assays are being examined with particulate samples. Preliminary data show that these cells are capable of engulfing particulate and do provide a more sensitive bioassay tool.

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Related Research

Research is ongoing to compare the response of the RAM in vitro to that of the whole animal as the result of inhalation of a variety of particulates from stationary sources.

SECTION 2

DEVELOP MORE RAPID AND SENSITIVE METHODS TO EVALUATE DOSE TO MAN

A. TASK TITLE:

Development of Test Systems to Assess Potential Toxicity and Neoplastic Transformation - Improvement of Scoring of Chemical Transformation of C3H/10T1/2 Cells (Substituted for) Development of Test Systems to Assess Toxicity and Neoplastic Transformation using Type 1 and Type 2 Alveolar Epithelial Cells *In Vitro*.

HERL/RTP TASK NO:	8153
GRANTEE:	University of Southern California
GRANT NO:	R-805208-01

Summary

The grantee plans to improve the quantitative oncogenic transformation by chemical carcinogens and score for transformation at earlier times so that the C3H10T1/2 mouse fibroblast system can be useful as a rapid prescreen for environmental pollutants. Individual compounds, as well as mixtures, will be studied. The use of single cells in individual dishes as the basic system will be improved, and the inhibitory influence of cell density on transformation frequency will be accurately quantitated. Transformed C3H/10T1/2 cells have a different morphology in the scanning electron microscope (SEM). This property will be used to develop an alternative assay for transformation and to determine at what time after carcinogen treatment cells become transformed. Other parameters of oncogenic transformation will be correlated with morphological changes observed by SEM and light microscopy. A test for environmental samples which may serve as promoters of carcinogenesis will be established.

Scope and Objectives

At the present time several systems have been developed for obtaining quantitative data on the oncogenic transformation of cultured cells by chemical carcinogens. It seems clear that such systems, although more complicated and tedious than a bacterial mutagenesis assay, may be more relevant as prescreens for carcinogenic activities. They also provide valuable test materials for studying the cellular and molecular mechanisms of chemical carcinogenesis; such information will be of practical value in providing means to prevent human cancer.

The grantee has developed a system for studying chemical oncogenesis in vitro with the C3H/10T1/2 mouse embryo fibroblasts. However, before this system can be validated as a potential prescreen by testing it for a larger number of carcinogenic and noncarcinogenic chemicals, additional research must be done to perfect its quantitative application.

The overall purpose of the present study is to perfect the quantitation of chemical transformation of C3H/10T1/2 cells in culture. The timetable and objectives under this grant will be: Year 1. Determine the optimal conditions for transformation of C3H/10T1/2 cells using the several criteria mentioned below. Year 2. Compare the quantitation of transformation by the optimal conventional methods and the single cell methods, and begin to screen known and unknown samples of environmental carcinogens with and without liver hemogenate-mediated activation, and environmental promoters with the purpose of validating the systems. Year 3. Continue validating the systems and screen unknown environmental samples provided by the EPA and other interested sources.

The perfection and validation of a system that determines and quanitates oncogenic transformation will produce the following benefits:

1) A reliable pre-screen for environmental carcinogens (or mixtures thereof) is one of the highest priorities in environmental surveillance.

2) Such a pre-screen can be widely applied to test large numbers of samples (pure compounds and mixtures) of environmental pollutants for their carcinogenic activity.

3) The determination if the oncogenic transformation of cultured animal cells is more relevant to human carcinogenesis than measuring mutagenesis in bacteria or other biological or biochemical parameters.

4) The cost, space, and manpower requirements to test individual samples would be expected to be less than 1% of those required to carry out adequate tests of carcinogenesis in living rodents.

Background and Approach

For many years the grantee has been interested to ascertain the cellular and molecular mechanisms for the initiation of cancer, particularly with polycyclic aromatic hydrocarbons (PAH) on the skins of mice. However, he recognized the severe limitations of working in vivo, and turned his attention to developing systems for the study of chemical carcinogenesis in vitro. During initial exploratory studies, the pioneer papers of Berwald and Sachs appeared, which clearly demonstrated that quantitative studies of carcinogenesis could, indeed, be carried out in cell cultures. Since then, the field has developed to the point where several systems have been developed, are in use, and are realizing their potential by providing useful tools both for the study of fundamental mechanisms and for potential primary screening systems for environmental carcinogens. This grant speaks primarily to the latter objective.

In order for the C3H/10T/2 mouse cell system to be perfected as a prescreen for environmental carcinogens, it is necessary that the transformation frequency (TF) be accurately quantitated. At the present time, this parameter depends entirely upon the method used for carrying out the transformation assay, the time interval before fixation and staining (which has been arbitrarily set at 6 weeks), and the method of scoring and counting of transformed colonies. The grantee has already obtained some data to indicate that the transformation frequency (TF) varies inversely with the number of cells plated. This needs to be repeated in a much more thorough way with particular attention to numbers of cells plated between 1 and 10. Another problem in quantitating the TF is that there is a migration of cells from one focus to form another. If such migration occurs, the TF obtained by counting foci at 6 weeks will be too high. This migration needs to be studied more thoroughly. The grantee has, as mentioned previously, arbitrarily fixed, stained, and scored for transformation at 6 weeks. He needs to determine how the number of transformed foci would vary with time between treatment and fixation, assuming that the problem of spreading of foci is solved. The major purpose of this grant application is to study these parameters so that the optimal conditions for elucidation of the TF can be determined. This is of tremendous importance in quantitative screening of environmental pollutants that may contain both strong and weak carcinogens.

Another problem with the cell culture system is that it is not known at what time malignant transformation takes place after a single treatment with a carcinogen. There are striking changes in the surface morphology of these cells on transformation, as seen in the scanning electron microscope (SEM). If such changes occur within a few days, it should be possible to greatly shorten the time of the transformation assay by use of the SEM as the primary method of scoring, instead of waiting for 6 weeks to score for transformed foci. This will be investigated both qualitatively and quantitatively. Finally, systematic investigations will be carried out on a series of additional parameters of transformation which may add to the precision of and decrease the subjectivity of scoring for oncogenic transformation. These parameters include: a) quantitation of the ability of a large number of nontransformed and transformed clones to grow in soft agarose as a means to determine loss of anchorage dependence; b) agglutination by plant lectins such as concanavalin A; c) acquisition of fibrinolytic activity; d) permeability to 2-deoxyglucose; and e) appearance of tumor-specific transplantation and embryonic antigens by lymphocyte-mediated cytotoxicity tests. In all cases, these parameters will be compared in individual transformed clones with their piled-up phenotype in foci, and with their ability to produce fibrosarcomas on inoculation into syngeneic mice.

The grantee has succeeded in applying the same principles of liver homogenated-mediated activation of polycyclic hydrocarbons and aflatoxins to produce mutations at the GHPRT locus in Chinese hamster V79 cells (D. F. Krahn and C. Heidelberger, Mutation Res. 46, 27, 1977). In research supported elsewhere, the grantee is applying this method of activation to oncogenic transformation of C3H/10T1/2 cells. The grantee will apply the same activation system in future years of this grant to the testing of environmental samples.

Research Accomplished

The grant has been in effect since October 1977. The grantee has investigated the time required to score the maximum frequency of transformation in the C3H10T1/2 mouse fibroblast system. Following a single treatment with 3-methylcholanthrene, cultures were scored for transformed foci from the 4th to the 10th week. Foci began to appear starting with the 4th week and remained approximately constant from the 5th to the 10th week. Scoring will be performed at 6 weeks as in previous studies. The grantee has sought a better way of scoring transformation frequency in the 10T1/2 system. Initial experiments with multiwell culture plates were unsuccessful due to detachment of confluent cell monolayers before individual colonies would be scored. The grantee will experiment with growing single cells on glass cover slips to permit direct observation with precise scoring of transformation frequency. Preliminary investigation of surface morphology of normal and malignant cells has begun.

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Related Research

In Vitro and *In Vivo*-*In Vitro* Systems for Determining Potential Carcinogenicity of Environmental Agents. In-House Task No. 8318 under Program Element 601F, Carcinogenesis.

B. TASK TITLE:

Enzymatic Characterization and DNA Binding of Carcinogens in Short-Term Bioassays

HERL/RPT TASK NO: 8156

GRANTEE: Columbia University

GRANT NO: R805482-01

Summary

A series of short-term bioassays including the Salmonella typhimurium (Ames) microbial mutagenesis bioassay and the several neoplastic transformation bioassays are being compared to in vivo tissue and human cells with respect to their ability to metabolize pro-carcinogens. This research will determine whether the carcinogen bound DNA adduct found in these test systems is similar to that found in intact human tissue. Enzymatic characterization of the carcinogen metabolizing capability present in these short-term bioassays is being conducted concurrently.

Scope and Objectives

The objective of this research is to determine if the activation of a known carcinogen and its covalent binding to DNA within the cell is qualitatively and quantitatively similar in several in vitro test systems to that which occurs in human cells and in the intact animal. The technical approach includes a comparison of:

A. isolated and characterized benzo(a)pyrene - nuclesoside adducts from DNA

B. microsomal enzyme activities

C. metabolite profiles

Emphasis in this project is on the carcinogen DNA adducts since this provides more definitive information on the ultimate carcinogen generated in each system and bound to DNA.

This work is being conducted in three phases. First, Salmonella typhimurium will be examined as it is utilized in the standard Ames Assay utilizing Arochlor 1254-induced rat liver microsomal S-9 activation. During the initial phase the following assays for neoplastic transformation will also be examined:

A. syrian hamster embryo system

B. C3H10T1/2 cell system

The second phase of this work will include studies with intact cells as metabolizers of carcinogens and the use of co-cultivation assays.

The third phase which has not been funded would utilize epithelial and human cell systems which are currently in the developmental of validation state.

Background and Approach

It is now recognized that animal bioassays are inadequate for monitoring the thousands of environmental agents which require screening as possible carcinogens. Several short-term *in vitro* tests for mutagenicity and carcinogenicity are currently being evaluated (by governmental agencies, private industry and various research groups). Since many environmental mutagens and carcinogens require prior metabolism or whole cells to exert their effects, some of these short-term assays use a rat liver microsomal fraction for activation of the chemical being tested. There is a paucity of information, however, on whether or not the activitation of a known carcinogen and its covalent binding to cellular DNA in these *in vitro* systems is quantitatively and qualitatively similar to that which occurs in intact mammalian cells, or in the whole animal. The objective of this proposal is to examine the modified DNA from cells used in these assays after exposure to the ubiquitous carcinogen benzo(a)-pyrene (B[a]P). The approach will be to incubate tritium labeled B[a]P with microsomes and *Salmonella typhimurium* tester strains, or with various mammalian cell lines. Cellular DNA will then be extracted, analyzed for radioactivity and fluorescense, and then hydrolyzed to nucleosides which will be analyzed by high pressure liquid chromatography, utilizing appropriate B[a]P-nucleoside derivatives prepared chemically as markers to determine the nature of the B[a]P-nucleoside adducts present. Parallel assays will be done for mutagenicity and transformation and of aryl hydrocarbon hydroxylase and epoxide hydratase activities. The data will be correlated with our results obtained in intact human tissues where it has been possible to determine the structure of the major adduct formed.

Research Accomplished

The DNA bound benzo(a)pyrene (B[a]P) adducts from the 10T1/2 oncogenic (neoplastic) transformation bioassay have been isolated and characterized. The major bound adduct is the 7, 8-dihydro diol 9, 10-epoxide of B[a]P. Further characterization of these adducts is in progress. This is also the major adduct in Syrian hamster embryo cells *in vitro*, bovine and human bronchial mucosa *in vitro*, and skin *in vivo*. This finding provides evidence that the *in vitro* 10T1/2 mouse cell oncogenic transformation bioassay metabolized B[a]P to the same major DNA bound adduct as do both an *in vivo* mouse system and an *in vitro* human organ culture system. The DNA bound B[a]P adducts are now being isolated from the *Salmonella typhimurium*/microsome (Ames) bioassay.

Bibliography

Papers produced as a direct result of these funds are in progress.

Related Research

EPA is currently funding the evaluation of several *in vitro* test systems to screen environmental chemicals for potential carcinogenic and mutagenic activity. In order to detect activity in chemicals which require activation, either a liver microsomal fraction is added to the test or cell systems are utilized which retain ability to metabolize carcinogens. It is important to know whether the activation in each of these systems metabolizes carcinogens by the same route and to the same ultimate carcinogen that occurs in whole animals and humans. This is crtical to the utilization of *in vitro* systems as valid predictors of responses in animals or human populations.

This project proposes to answer these questions in the short-term tests currently being either employed or evaluated by EPA.

C. TASK TITLE:

Development of Bioindicators to NO_2 and SO_2 Exposure

HERL/RTP TASK NO: 9172

CONTRACTOR: Southwest Foundation for Research and Education

CONTRACT NO: 68-02-2279

Summary

During the initial year of this investigation, efforts were concentrated towards screening for potential bio-indicators centering around exposures to SO_2. Areas of research pursued included effects of this pollutant on lymphocyte responsiveness, circulating plasma neutral lipids, biogenic amines and hormones, in addition to protein alterations induced by inhalation.

Based on findings in these preliminary studies, it was decided that the approach showing most promise for continuation in years II and III would be a thorough evaluation of altered immunological function as well as circulating biogenic amines and hormones with focus on dose and temporal effects. The assessment of lumphocyte function through determinations of mitogen responsiveness was to be pursued. It was shown that *in vitro* treatment of globulin with bisulfite produces structural alterations of protein which could hopefully be used as immunogen for attempting to produce and isolate specific antibodies.

Short-term continuous exposure studies designed to examine acute and latent effects in adult male Wistar-Lewis rats as a result of varying atmospheric concentrations (2.5, 5 and 10 ppm) of SO_2 and NO_2 are continuing. Assays were performed on blood samples obtained from animals in experimental and control groups at each of several time intervals during both exposure and recovery. Separate experiments for the determination of effects on normal diurnal variation of corticosterone were conducted. Initial experiments have now been performed for each of the exposure levels and will be duplicated for evaluation of pooled data.

For comparison with accumulating evidence that short-term exposures have different effects than the same dose over longer periods, chronic intermittent exposures will be conducted during the final year of this contract. In an efforts to stimulate the normal work week of the human, exposures will run twelve hours per day, five days per week for five weeks.

Scope and Objective

The intention of this research effort is to examine the biochemical action of NO_2 and SO_2 on lung metabolism and to develop alterations that occur into a dose-response bioindicator. Phase I: Biochemical investigations of lipid and protein metabolism of both the lung and distal organs after nitrogen dioxide and sulfur dioxide exposure. Phase II: To measure release of bioactive compounds and/or their metabolites after NO_2 and SO_2 exposures. To develop any detected alterations into bioindicators.

Background and Approach

The current standard for NO_2/SO_2 rests mainly on epidemiological studies and their toxicity is less well defined than for the other major air pollutants. Concern exists that exposure to these pollutants resulting from sources such as automobile exhausts, power plants, or burning of coal may create a major health hazard. Studies have previously shown that edematous and hemorrhagic lungs are produced by nitrogen oxide irritants involving primary reaction with lipid and proteinaceous material and causing damage to the lung cells and surrounding capillaries. Acute toxicity of occupational related nature has occurred in welders, firemen and nitric acid plant workers. Additionally *in vitro* experiments have demonstrated alterations in glucoproteins of macrophage and lymphocyte cell membrane. Such changes may affect the immune response and/or produce disturbances in hormonal action at the cellular level.

Inhalation of SO_2 has resulted in absorption and penetration into the systemic circulation. This has been demonstrated utilizing $^{35}SO_2$, the ^{35}S of which diffuses through the lung into the circulatory system and is distributed throughout the body. Interaction of these gases with glycoproteins at the cellular level could potentially alter neurohormonal mechanisms controlling endocrine systems, particularly the pituitary-adrenal axis. Disruption of either the CNS or target organ cellular membrane could alter the cyclic hormone cascade, which might be demonstrated through changes in ACTH and/or glucocorticoid pattern. It was then suggested that plasma ACTH and corticosterone be examined in light of their role as important chemical mediators.

It was proposed, therefore, to evaluate the effect of exposure to high level sub-lethal doses of NO_2/SO_2 through measurement of chemical parameters of blood. Initial screening should include biogenic amines and their metabolites, corticosterone and ACTH, CAMP, prostaglandins, histamine, and neutral lipids. Effects of dose and duration should then be determined for those indices showing and effect.

The second category under investigation is that of immune response. *In vitro* studies of blast transformation by circulating blood lymphocytes are being conducted for detection of alteration of responsiveness possibly contributing to compromised immunocompetence. Assessment of lymphocyte blastogenesis through mitogen stimulation is being examined using PHA, ConA and LPS. Alteration of ability to respond to mitogen stimulation is an indication of detrimental influence on defense mechanisms.

Research Accomplished

As of this date the first two years of investigation under this contract have been completed. Major milestones planned and achieved include the screening of potential bioindicators in rats subjected to sulfur dioxide exposure at high sub-lethal levels and selection of appropriate assays. The effects of five-day exposures of rats to 5 ppm of both SO_2 and NO_2 have been evaluated in two separate groups. Initial exposure to 2.5 and 10 ppm for each of these pollutants have been conducted. They will be repeated subsequently.

Available data indicate a possible acute effect of the two gases on the serotonergic system at certain time points during exposure. In addition, a residual effect appears to be exerted by 2.5 and 5 ppm SO_2 as evidenced by elevated mean levels of serotonin during the recovery period. These observations were not augmented by increasing the SO_2 level further. Exposure to 5 ppm NO_2 revealed changes interpreted to indicate primary exposure response of serotonin and epinephrin during the experimental testing followed by a secondary recovery upon termination of exposure. More labile effects were exhibited in the sympathetic system. Plasma corticosterone was significantly depressed at 14 days post exposure to 10 ppm SO_2 while ACTH was elevated. Negligible effects of both gases were seen in other parameters of cAMP, histamine, lipids and prostaglandin.

The most striking result of immunological indicators to date was that of a depressed mitogenic response to Con A immediately after exposure to 5 ppm and remaining through 28 days after the highest dose of SO_2. At 2.5 ppm no lasting alterations were found. Decreased response was observed for all mitogens tested after 24 hours exposure to 10 ppm and 120 hours to 5 ppm NO_2. There is some indication that the observed changes might be more persistent at the higher dose level.

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Related Research

NASA contract recently awarded to Dr. Irving Geller of Southwest Foundation and project director of Contract #68-02-2279 for a study of SO_2/NO_2 Behavioral Effects. (Personal Communication)

D. TASK TITLE:

In Vitro Screening of Selected Air Pollutants for Potential Carcinogenicity.

HERL/RPT TASK NO: 8190

CONTRACTOR: Microbiological Associates

CONTRACT NO: 68-02-2271

Summary

A tiered approach to the screening of environmental agents has been developed and implemented. This approach includes the use of bacterial mutagenesis, mammalian cell mutagenesis and neoplastic transformation bioassays. Agents which have been tested in this tiered system include pesticides, asbestos-like fibers, polycylic aromatic hydrocarbons and insoluble metal particulates.

This contractor has studied the feasability of coupling metabolic activation systems (e.g. rat liver homogenate or primary embryo cells) with mammalian cell mutagenesis and oncogenic transformation bioassays and have developed these combined systems which give increased sensitivity to the detection of genotoxic agents. The most significant accomplishment has been the development of a bioassay which simultaneously measures mutagenesis and oncogenic transformation in mammalian cells with the addition of exogenous metabolic activation. This system is now in the validation stage and will be an important tool in the screening of large numbers of environmental agents.

Scope and Objectives

The scope of this contract is encompassed in three subobjectives:

A. To screen for the potential mutagenic and carcinogenic activities of selected air pollutants and environmental chemicals provided by the Environmental Protection Agency.

B. To determine the feasibility of combining *in vitro* mammalian cell bioassay systems with various mammalian activation systems (both intact cellular and sub-cellular) to enhance the sensitivity to low concentrations of biologically potent chemicals or chemicals of poor biological activity.

C. To develop an assay which measures simultaneously both mutation and oncogenic transformation.

The tiered approach being employed to evaluate the biological activity of the compounds supplied by the EPA is being developed along the following lines. The mutagenic potential of each chemical agent is first determined in the Salmonella typhimurium mutagenesis assay developed by Ames. These compounds which prove to be negative in this test are then retested in the presence of a source of mammalian metabolism in the form of the 9000 x g supernatant (S-9) derived from rat hepatic tissue. Agents which are found to be positive mutagens in the bacterial prescreen are then assessed for their cytotoxic and mutagenic potential to establish *in vitro* mammalian cell lines. Those cell systems presently being evaluated include the V-79 Clone 8 (V-79) Chinese hamster lung line the BALB/c 3T3 Clone A-31-1 (3T3-1) mouse line and the mouse C3H10T 1/2 Clone 8 (10T 1/2) line.

The incorporation of mammalian metabolic activity in order to increase the sensititivity of these assay systems both in terms of detection of weak carcinogens or low doses of strong carcinogens, as well as detection of diverse classes of carcinogens, are being developed along three lines:

A. Cocultivation of target cells with rodent S-9 fractions

B. Cocultivation of target cells with freshly derived rodent hepatocytes.

C. Cocultivation of target cells with X-irradiated mitotically treated metabolically active hamster embryo cells.

Preliminary assessments are being made of the inherent levels of polycyclic aromatic hydrocarbon metabolizing activity (in terms of aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH)) in each of these cellular and sub-cellular sources of enzyme activity. The inherent cytotoxicity of these preparations for the *in vitro* model cell systems are also being evaluated. Incorporation of these preparations into mammalian mutation and transformation assay systems will be done with the appropriate S-9's as determined by the above preliminary tests.

Evaluations are being made as to the feasibility of utilizing one or more mammalian cell systems, in the presence or absence of an exogenous source of metabolic enzymatic activity, to assay for the chemical induction of ouabain-resistance mutation and transformation.

Background and Approach

From the time Sir Percivall Pott recorded in 1775 his observations of "soot-wart", the scrotal and testicular cancer of chimney sweeps of Old England, enviromental chemicals have been recognized as potential etiological carcinogenic agents to man. The earliest reported experimental induction of animal tumors, however, did not appear until the twentieth century when Yamagiwa and Ichikawa produced carcinomas on the skin of rabbits painted with chimney soot or coal tar. Finally, approximately 15 years later, Kennaway and Hieger and Cook obtained extracts of benzo(a)pyrene and 1,2,5,6-dibenzanthracene from such sources as coal tar, and found these polycyclic hydrocarbons to ellicit neoplasms *in vivo*. Several other classes of chemical agents were also soon recognized as potential environmental carcinogens, including aminoazodyes, aromatic amines, nitrosamines, carcinogenic metals, etc. Polycyclic aromatic hydrocarbons, benzo(a)pyrene, in particular (a known potent carcinogen), has been shown to be expelled into the atmosphere in the range of 1320 tons per year in the United States alone. Furthermore, the smoke of 40 cigarettes has been reported to deliver 1 g benzo(a)pyrene. In view of these data and the fact that as much as 90% of all human cancers have been attributed to chemical agents, an appropriate method by which to screen such environmental chemicals for their carcinogenic potential is of utmost importance. Considering the vast number of compounds that would need to be tested, the sole use of *in vivo* animal systems becomes an impossibility in terms of finances and trained personnel. In addition, tumorigenicity *in vivo* is affected by the species of animal and its genetic background, presence of pathogens in the strain, promoting agents in the diet, drug metabolizing enzymes, dosage and duration of treatment, route of inoculation, age and life span of the animals, and hormonal variations. Reliable and reproducible *in vitro* test systems offer the following advantages as a preliminary screen:

1. Relatively low cost per compound; 2. a test which usually can be initiated and completed in less than three months; and 3. microgram quantities of rare or expensive compounds could be tested, whereas much larger quantities are needed for bioassay in animals.

Compounds which transform the cells *in vitro* can then be further evaluated for *in vivo* carcinogenicity using the long-term animal systems.

Major tissue culture transformation systems currently available include the hamster embryo system, the C3H 10T1/2 clone 8 system, and the BALB/3T3 systems. Since many of the enzyme systems which can change the different chemicals to their proximate or ultimate carcinogenic forms are unknown or may be absent in the cell culture, more complex cell- or enzyme-mediated assays need to be developed.

Research Accomplished

A. Bacterial Mutagenesis--

1. Screening-- A number of various different types of samples were tested according to the procedure of Ames including the pesticides Captan, Folpet, and asbestos-like fibers Amosite and Chrysotile. The former agents were positive while the latter negative in this test system. An insoluble carcinogenic metal, nickel subsulfide, was also tested and was negative.

2. Developmental-- Studies have been initiated to examine the (AHH) aryl hydrocarbon hydroxylase (a carcinogen metabolizing enzyme) activity of different S-9 preparations and to relate the AHH activities to the induced mutation frequency of *S. typhimurium* tester strain TA 100. The S-9's were generated from Fischer 344 male rat hepatic tissue, either from control rats or from rats induced by intraperitoneal injection with 500 mg Aroclor-1254 per kg body weight once 48 hours prior to sacrifice, or with 50 mg phenobarbital (PB) per kg body weight once each day for five days prior to sacrifice. AHH activity was monitored by the conversion of benzo(a)pyrene (B[a]P) to the

phenol, 3-OH B[a]P. Mutation of TA 100 was monitored by reversion from histidine auxotrophy to prototrophy, using 6-aminochrysene as the mutagen. Cytotoxicity determinations were also run in parallel in order to develop a mutation frequency. In terms of AHH activity, Aroclorinduced S-9 gave the highest metabolic conversion of B[a]P to 3-OH B[a]P. PB-induced S-9 had about 1/3 the activity of Aroclor-S9. Control (noninduced) S-9 showed about 1/5 the AHH activity of Aroclor-induced S-9. However, the mutation frequency induced in the presence of each of these S-9 preparations did not parallel AHH activity; PB-induced S-9 gave the highest mutation frequency per AHH.

B. Mammalian Cell Mutagenesis and Oncogenic Transformation--

The feasibility of coupling mammalian metabolic activation systems with *in vitro* mammalian cell bioassay systems was explored. The following two mammalian bioassay systems were cultivated with induced rat liver homogenate and benzo(a) pyrene: Chinese hamster cells V-79 (mutagenesis) and BALB 3T3 mouse embryo fibroblasts (cytotoxicity, mutagenicity, and oncogenic transformation). In all cases an increase was observed in the biological activity of each of the systems incubated with the homogenate and B[a]P when compared to B[a]P alone indicating that the liver homogenate activated the carcinogen to forms which interacted with the cells.

Other metabolic activation systems were also examined. These were whole cell preparations which included primary Syrian hamster embryo cells, primary rat hepatocytes, and various strains of irradiated embryonic cells.

C. Amplification assay for enhanced detection of chemically-induced morphological transformation--

The standard 3T3 and 10T 1/2 focus transformation assays have proven to be routinely reliable in ascertaining the neoplastic potential of model chemical carcinogens. There are certain limitations in both systems; one of these is the fact that similar treatment of target cells with potential carcinogens does not always result in comparable transformation responses. In addition, not every replicate dish treated with a given carcinogen gives rise to Type III transformed foci. This failure in uniformity of response suggests the possibility that certain potential carcinogens may be incorrectly scored as negative if they fail to induce focus formation in the standard assay.

In pilot studies with 3T3 cells it has been observed that formation of morphologically transformed foci in dishes generated from a single subcultivation of cells (treated with transforming doses of carcinogen) derived from dishes which showed no foci after the standard four week assay. Based upon these results a procedure for amplifying the presence of phenotypically transformed 3T3 cells potentially capable of forming Type III transformed foci, has been devised and validated with standard carcinogens.

D. Development of a Simultaneous Mutagenesis and Transformation Bioassay with Exogenous Metabolic Activation--

Another approach which was employed with the 3T3 cell system was to simultaneously monitor both chemically-induced mutagenesis (at the *oua*^{r} locus) and transformation (Type III focus formation). These studies were carried out using 3-methylcholanthrene (3-MC) as the mutagen/carcinogen in the absence of any exogenously supplied metabolic enzyme (S-9) preparation.

To date, the model chemicals examined in the standard plate assay have included 3-methycholanthrene (3-MC), N-methyl-N'-nitro-N-nitrosoquandiine (MNNG) and 6-aminochrysene (6-AC). Each time morphological transformation (Type III foci) was observed, mutagenesis at the ouabain-resistance (oua^r) locus was observed, and vice versa, when 3-MC or MNNG were tested. Using the 3T3 system, an induced mutagenic event coincided with a transformation event with each of 3 classes of chemicals surveyed. Also a number of pesticides were tested in these test systems for transforming ability and mutagenesis. These agents included Folpet and Captan, two fungicides.

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Related Research

In Vitro and *In Vivo-In Vitro* Systems for Determining Potential Carcinogenicity of Environmental Agents. In-House Task No. 8318 under Program Element 601F, Carcinogenesis.

Development of Test Systems to Assess Potential Toxicity and Neoplastic Transformation - Improvement of Scoring of Chemical Transformation of C3H/10T1/2 Cells (Substituted for) Development of Test Systems to Assess Toxicity and Neoplastic Transformation using Type 1 and Type 2 Alveolar Epithelial Cells *In Vitro*. Grant No. R-805208-01, University of Southern California, Task No. 8153, Program Element No. 625F, Energy.

E. TASK TITLE:

In Vitro Screening of Selected Air Pollutants for Potential Carcinogenicity Using Microbial Systems.

HERL/RTP TASK NO: 8191

CONTRACTOR: Research Triangle Institute

CONTRACT NO: 68-02-2724

Summary

Solvent fractionation is used to separate components of particulate

matter in air collected by means of a Battelle Massive Volume Air Sampler. The crude material and resulting fractions are subjected to bioassay (mutagenesis) via a *Salmonella typhimurium* reverse mutation detection system. Positive results form the basis for chemical composition determinations, further fractionation and additional bioassay. High pressure liquid chromatography (gel permeation, adsorption and partition modes) is used for additional separation with chemical structural analysis accomplished via chromatographic retention time determinations, spectral (UV adsorption, fluorescence excitation and emission) comparison with standards, and direct probe mass spectrometry coupled with a computerized mass spectral search system. Volatile vapors and aerosols are collected from the atmosphere using sampling cartridges containing a sorbent material. After collection the materials are thermally desorbed for chemical identification and bioassay purposes.

Scope and Objectives

Industrial emissions and wastes include agents which are known to be mutagenic/carcinogenic and thus constitute a health hazard to man in the form of cancer, birth defects and heritable disease. In addition to the known agents there are probably present many undetermined mutagenic/carcinogenic substances as well.

Clearly, the most reasonable approach to the evaluation of complex environmental samples is a judicious combination of chemical identification, fractionation and biotesting. It is presently not possible to specify a detailed protocol which incorporates all these aspects and which is maximally efficient and cost effective. Thus, the purpose of the proposed work is to develop such a protocol and in so doing, define a minimal biological and chemical methodology which will function as an effective screen for potential mutagenicity/carcinogenicity (and other related hazards) of complex mixtures occurring as air pollutants.

Background and Approach

Recent work suggests that a limited set of mutagenesis tests may be the best of presently available methods to use as indicators for the most serious human effects. These have the ability to indicate, with mutation as the test end point, a variety of possible effects that involve the disruption of biochemical genetic mechanisms which are shared generally by all forms of life.

Recently there have been demonstrations of correlation between carcinogenesis and mutagenesis (Miller and Miller, 1971; Ames, 1972) and it has been implied that most cancers may be due to somatic mutations (Ames, 1972; Ames, *et al.*, 1973). The numbers of compounds that have been demonstrated to be mutagenic and also carcinogenic and/or teratogenic (Miller and Miller, 1971; Kalter, 1971) recommend mutagenesis tests as indicators.

Given that mutagenesis tests deserve consideration for evaluating the biological impact of contaminating substances in the environment, there remains the selection of particular mutagenesis tests and adapting them to the study of mixtures of airborne materials. One of the most promising current methods is the Salmonella typhimurium histidine reverse mutation system described by Ames, McCann and Yamasaki (1975). Already more than three hundred chemicals have been evaluated for mutagenic activity with this system.

There are two simplistic approaches which could be used for the evaluation of mutagenic potential of crude air samples. One is to perform very thorough and exacting chemical analyses. Then on the basis of mutagenesis tests of the pure compounds identified by the analysis, decide whether or not a hazard exists. This approach is not very satisfactory because it is the mixture to which humans are exposed and not the individual components, and there may be interactions which make the mixture more or less active than the individual agents evaluated separately. There is, too, the possibility of sample matrix effects which may limit the availability of particular agents to the biological test material. Another simplistic way of approach would be to directly subject the crude materials to biotesting without regard for chemical analysis. Also very unsatisfactory, this would allow hazardous substances, temporarily masked by inhibition, or interaction, etc., to pass unnoticed. Additionally, if there are effects other than mutagenicity for some agents in the mixture, it is quite possible for the effect of one compound to prevent the detection of another. Thus, a toxic effect of one agent could prevent (because effective concentrations could not be reached) the detection of mutagenicity of another. This task represents a compromise between these two approaches such that a limited but effective fractionation scheme will be performed in conjunction with a simplified but complete bioassay protocol.

The bioassays will depend most heavily on three strains of Salmonella typhimurium (TA-1535, TA-1537, and TA-1538) which are histidine deficient and with which reversion to prototrophy is indicative of mutation. Testing will be done both with and without the inclusion of a metabolic activation system for the detection of directly and indirectly acting mutagens.

The methods selected for the collection of pollutants from ambient air will provide a representative continuum from the more volatile organic vapors and aerosols to the semi-volatile and non-volatile compounds associated with particulate. Sample collection strategy will be designed to allow the collection of sufficient quantities of materials by each collection method. The two collection methods proposed are the sampling cartridge containing a sorbent material (Tenax GC) for the concentration of the volatile vapors and aerosols from the atmosphere and the Battelle Maxi Air Sampler for the collection of particulate matter.

The volatile vapors trapped on Tenax GC sampling cartridges will be removed by thermal desorption to recover the trapped organic vapors for subsequent bioassay or for chemical characterization by high resolution gas chromatography/mass spectrometry/computer analysis. Thermal desorption systems of varying design have been described for recovering trapped vapors with their subsequent analysis by glc and glc/ms. The Contractor proposes to recover the adsorbed trace organic vapors utilizing an inlet-manifold developed at RTI (EPA Contract No. 68-02-1228) Pellizzari, 1974).

The fractionation sequence for particulate is divided into three levels of separation. Initial separation of the particulate sample into groups containing the inorganic matrix, polar organics, and non-polar organics occurs in the first level. Beyond this point, primary emphasis is placed on the biological evaluation of the non-volatile organic constituents of the particulate sample. Further evaluation of the inorganic fraction with respect to effects on the bio-availability of organic components is considered to be an ancillary objective in this proposal.

The second level of fractionation divides the drude organic mixtures into general chemical classes containing polar and non-polar acidic, basic and neutral components, respectively, with the non-polar neutrals being further sub-classes into parafins and aromatics. All fractions produced in levels 1 and 2 will be subjected to bioassay in order to evaluate the existence of synergistic relationships between constituent classes, including the inorganic matrix.

Level 3 involves the separation of the class mixtures from level 2 into their respective components as required for the qualitative and quantitative analysis of mutagenic agents. High pressure liquid chromotography (HPLC) will be utilized extensively at this level.

Although the initial class separation and subsequent resolution of individual components is a requirement for the evaluation of synergisms and identification of active components, considerable shortening of the fractionation scheme may be possible for the routine quantitation of specific compounds in the particulate sample. This aspect will be examined as part of the quantitative evaluation.

Research Accomplished

A "well method" for mutagenesis screening in a modified Ames test has been validated using known mutagens (2-anthramine, 2_acethlaminofluorene, benzo(a)pyrene, B-naphthylamine, sodium azide, wuinacridine hydrochloride, 9-aminoacridine and 2-nitrofluorene) with five strains of *S. typhimurium*. The "well test" proved as good or better than spot and disc tests and compared favorably with the pour plate test. The principal advantage is a greatly reduced sample size. The protocol for S-9 microsome preparation has been finalized. One time induction using 500 mg Arochlor/kg body weight proved superior to multiple injections at lower doses.

The chemical fractionation scheme for ambient particulate samples has been modified to reduce the total number of fractions to six as compared with the 13 originally produced with larger amounts of material in each fraction. The efficacy of the scheme was assessed by subjecting a mixture containing known amounts of compounds to the partition procedure. Recoveries were determined gravimetrically. TLC scans indicated no "spill over" of compounds into other fractions.

Using known mutagens, tests of the effects of varying microsome concentration, bacterial concentration, and histidine concentration were conducted in both pour and well procedures. Histidine concentrations of 20-40 micrograms/plate appeared to be the optimum. Variation of bacterial concentration in the range of 1.00 - 1.30 (OD at 420 nm) has little or no effect. Microsome concentration (.08 - 2.0 mg/plate) markedly affected response. Work is continuing to devise a mechanism to confine vapors and gases in such a way that they can be released quantitatively into the mutagenesis assay.

One proposed approach for the Ames testing of air vapors involves the transfer of vapors from Tenax cartridges (as collected in the field) to activated charcoal. The charcoal with the adsorbed vapors would then be placed in an agar well and mixed with a solvent that would desorb and disperse the vapors into the culture medium. Critical to this approach is the choice of desorbing solvent. Nine potential solvents were tested for compatibility with the Ames procedure. Of these only 2 were acceptable in terms of toxicity in relation to volume, DMSO and ethylene glycol.

It appears that DMSO is the only solvent that is both compatible with the Ames assay and capable of releasing significant quantities of carbon-adsorbed material such as EDB. Even in DMSO, a volume of 0.5 ml probably represents an upper limit in regard to what can be tolerated in the bioassay procedure. Because of these difficulties, the analytical/bioassay approach for vapors based on the use of carbon adsorption bears re-examination.

Other experimental approaches are under consideration. The most promising to date involves transfer of the vapors from Tenax cartridges into hollow-fiber filters (2000-50,000 molecular weight retention) which can be sealed at the ends and incorporated into the agar plates.

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Related Research

NIEHS/EPA Collaborative study on mutagenicity of ambient air using *Tradescantia* plant system in a mobile van.

F. TASK TITLE:

Develop Cellular Model System to Determine Cytotoxicity from Alternate Energy Sources

HERL/RTP TASK NO: 8193

CONTRACTOR: Dr. William E. Bowers, Rockefeller University

CONTRACT NO: 68-02-2426

Summary

A quantitative *in vitro* bioassay system to evaluate the effects of pollutants on lymphocyte cytotoxic activity is being developed.

Scope and Objective

The objective of this research is to develop a quantitative model system to determine the effects of *in vitro* pollutant exposure on lymphocyte cytotoxic activity. This will involve identification of the bioenergetic and biosynthetic pathways believed to be of importance to cytotoxicity and determination of the nature of any substance elaborated by lymphocytes involved in foreign cell destruction. After development of the model system, its suitability for use in environmental toxicity studies will be evaluated.

Background and Approach

The immune system has a variety of defensive functions against both infectious and neoplastic disease. The development of new energy processes raises the possibility that some fossil fuel emissions may have the potential of being immunosuppressive agents. However the large number of different substances which may be emitted calls for a screening research approach that will rank toxicity for later *in vivo* testing. Due to the complexity of the immune system, one of the better approaches to an *in vitro* study is to investigate effects on lymphocytes which are sensitive to the action of a variety of treatments.

The general features of lymphocyte physiology fundamental to the development of useful bioassasys are the following: small lymphocytes transform into large-sized lymphocytes as a result of their encounter with tumors and with foreign cells or as a response to stimulation by antigens and mitogens. These transformed lymphocytes then divide several times to give rise to progeny which carry out specific processes. For example, the progeny which result from the division of lymphocytes transformed by tumor or allogeneic cells are capable of killing specifically the tumor or allogeneic cells. Thus, transformation lies on a direct pathway to the production of effector cells having specific functions, and the entire sequence of events occurring *in vivo*--recognition of foreign surfaces, transformation, division, and production of specifically cytotoxic progeny--can be reproduced *in vitro*. Such an *in vitro* system makes an ideal object for the study of cytological and biochemical changes accompanying the production of cytotoxic lymphocytes and for the biochemical processes essential for the killing of tumor or allogeneic cells. Two of the important aspects of this system, namely transformation and cytotoxicity, will be made the subject of bioassays.

Research Accomplished

To date, a lymphocyte transformation and cytotoxicity assay has been established. The methodology has been improved by interfacing cell-sizing instrumentation to a microprocessor unit which will permit large numbers of samples to be evaluated sensitively and accurately. An automated method for determining ^{3}H-uridine incorporation into transforming lymphocytes has been developed. This will allow assessment of effects on earlier biochemical events in lymphocyte transformation, and it will complement the already developed method for measuring ^{3}H-thymidine incorporation. Work is progressing on identification of biochemical events associated with lymphocyte transformation and cytotoxicity. When the bioassay developmental phase is completed, pollutants will be added to the system.

Bibliography

None

Related Research

To my knowledge this investigator is not being funded by any other source to conduct the same research. The particular field of immunology involved in this project is subject to intensive investigation by the scientific community. Therefore, while I am not familiar with any duplication of effort, the possibility does exist.

G. TASK TITLE:

Development of Automated Behavioral Testing Methodologies for the Study of Coal Conversion and Utilization Products.

HERL/RPT TASK NO: 8155

TASK TITLE:

Application of the Automated Behavioral Testing System to Monkeys Exposed to Coal Conversion Pollutants - RFP-B116

HERL/RTP TASK NO: 8165

CONTRACTOR: Iowa State University

CONTRACT NO: 68-02-2288

Summary

The nervous system is a target organ for a number of substances which are emitted from energy related sources. Until recently little research has been specifically aimed at identifying neurotoxicity, especially as it relates to subtle behavioral effects brought about by chronic low-level exposures. The computer-automated pattern recognition and operant testing paradigms developed under the energy research program have proven to be behavioral testing procedures highly sensitive to the effects of low level d-amphetamine exposure in *Macaca fascicularis*. It is expected that these procedures will be invaluable in determining the neurotoxic effects of a number of energy related pollutants on complex bahavioral processes. Further, the primate appears to be an excellent animal model for examining the neurobehavioral effects of psychoactive drugs, and should represent the animal of choice when examining the effects of neurotoxicants on complex behavioral processes.

Background

The nervous system is a target organ for a number of substances which are emitted from energy related sources. For example, it is estimated that approximately 600 million tons of coal are consumed yearly in the United States. Of this total, in excess of 360 million tons are burned in central power plants. Several investigators have documented the presence of many neurotoxic trace elements (nickel, lead, cadmium, manganese, mercury, thallium and tin) in coal. Although many of these toxic trace elements are present in small quantities in coal, the extremely large volume of coal burned yearly can result in the mobilization of significant quantities of these elements into the environment.

Although toxicologists have for some time recognized the commonality of the nervous system as a target organ for a variety of toxic agents, until recently very little research has been specifically aimed at identifying neurotoxicity, especially as it relates to subtle behavioral effects brought about by chronic low level exposure. In the field of behavioral toxicology a particular need has existed for the development and validation of testing methods which are sensitive indicators of low level exposure, specifically long-term low level exposure. This need for the development and validation of testing methodologies formed the basis for the initiation of the present tasks.

Scope and Objectives

The main thrust of the research effort described in these tasks centers around the following objectives: (1) the use of the non-human primate as the experimental animal, and (2) the development and validation of computer based behavioral testing procedures.

Much of the current effort in methods development has focused on the use of the rodent as the test subject. The point to be made here is that a need exists for primate research in the area of behavioral toxicology. For example, the highly developed central nervous system and complex behavioral patterns make the primate highly suitable for some types of testing. Only recently have researchers begun the task of examining the effects of CNS toxicants on complex behavioral processes in animals. It is imperative that the effects of neurotoxicants on such complex behaviors as: perception, learning, storage and recall of information, the use of language, social interaction, development, motivation, and emotion be understood. It is only in the non-human primate that some of these processes are observed. One of the behavioral paradigms developed under Phase 1 of this effort examines a spectrum of complex behavioral activity patterns in the primate. This technique has been successfully used in behavioral pharmacology (and recently in behavioral toxicology) but these studies have employed the rat as the test subject. A second paradigm developed under Phase 1 research examines the ability of the primate to store and recall stimulus information over a long time interval--a task too difficult for a rat to perform.

The testing procedures referenced above have been employed in a non-automated environment. However, this application of these procedures imposes severe limitations on what are otherwise very informative behavioral testing methods. The computer automation of these tests would make practical the use of these and related procedures in screening a number of energy related toxicants for neuro-behavioral effect.

The objectives of these research tasks were therefore: (1) to develop computer-automated behavioral testing methods using the primate, (2) to validate these methods using pharmacological agents with known psychoactive properties, and (3) to apply these methods to toxicity testing of candidate pollutants.

Research Accomplished

Currently work on Task 8155 is scheduled for completion on 31 July, 1978, with work on Task 8165 to begin 1 August 1978 with completion scheduled for 31 July 1980.

To date, the computer automated pattern recognition system for the assessment of neuro-behavioral changes in the spontaneous behavioral activity of primates is complete. Testing of this system with primates exposed to a stimulant drug (dextro-amphetamine) are completed, and tests with a depressant drug (chlorpromazine) are currently being conducted.

The objective of developing a computer-automated pattern recognition system has been achieved. A computer system equipped with three closed circuit video cameras has been programmed to identify 40 separate primate behavioral acts. In addition to identifying each act, the system is capable of determining the frequency, duration and sequence of occurrence of each behavioral act. To a point, a "seeing computer" has been substituted for the human in the task of observing and classifying complex primate behavioral activities. This computer system has proven to be a highly reliable observer of primate behavior when compared to similar observations performed by trained human observers. Recent comparisons between the computer system and trained observers, show that the computer and the human observers agree in their observations approximately 85% of the time. However, when disagreements do occur, the human observer invariably agrees that the computer's initial observation was correct. In short, the computer is not only a more reliable observer from one occasion to the next than the human observer, it is generally a better observer.

Tests of the computer pattern recognition system using primates exposed to the stimulant drug, d-amphetamine, have produced some surprising and very encouraging results. Initial data analysis indicates that the computer pattern recognition system is highly sensitive to amphetamine produced changes in primate behavior. For example, if the computer system is programmed to identify the location of the primate within it's environment, rather dramatic differences are observed in the behavior of amphetamine exposed monkeys compared to controls. Figures 1 - 4 illustrate where, in a test cage, monkeys are located at each 1/2 second interval during the 13-minute observation period. It can be clearly seen that amphetamine, as dosage increases, causes a rather severe limitation in which parts of it's environment an amphetamine exposed monkey will occupy. This effect is clearly evident at the very low exposure of 0.11 mg/kg. In addition to constraining where the monkey goes in its environment, amphetamine also causes a dose related decrease in the total number of behavioral acts initiated by exposed monkeys during the observation period (hypoactivity). Early analysis also indicates that amphetamine causes the behavior of monkeys to be less structured than that of control animals. Sequences of ordered pairs, triplicates and quadruplicates of behavioral acts occur less frequently, overall, in amphetamine exposed primates. The duration of behavioral acts is also altered, however, this data is currently being analyzed and the specific nature of the alterations have yet to be determined.

Tests of the amphetamine exposed primate's ability to respond to stimuli over delay intervals varying from 0 to 24 seconds also shows dramatic changes. Data presented in Table 1 clearly show that monkeys exposed to 0.33 or 1.0 mg/kg d-amphetamine stop responding on a delayed response test. This task is easily mastered under control conditions.

The data collected thus far demonstrates that the computer-automated behavioral paradigms are clearly sensitive to amphetamine produced changes in primate behavior, and that rather dramatic alterations occur at very low levels of exposure.

It is expected that these computer-automated procedures will be

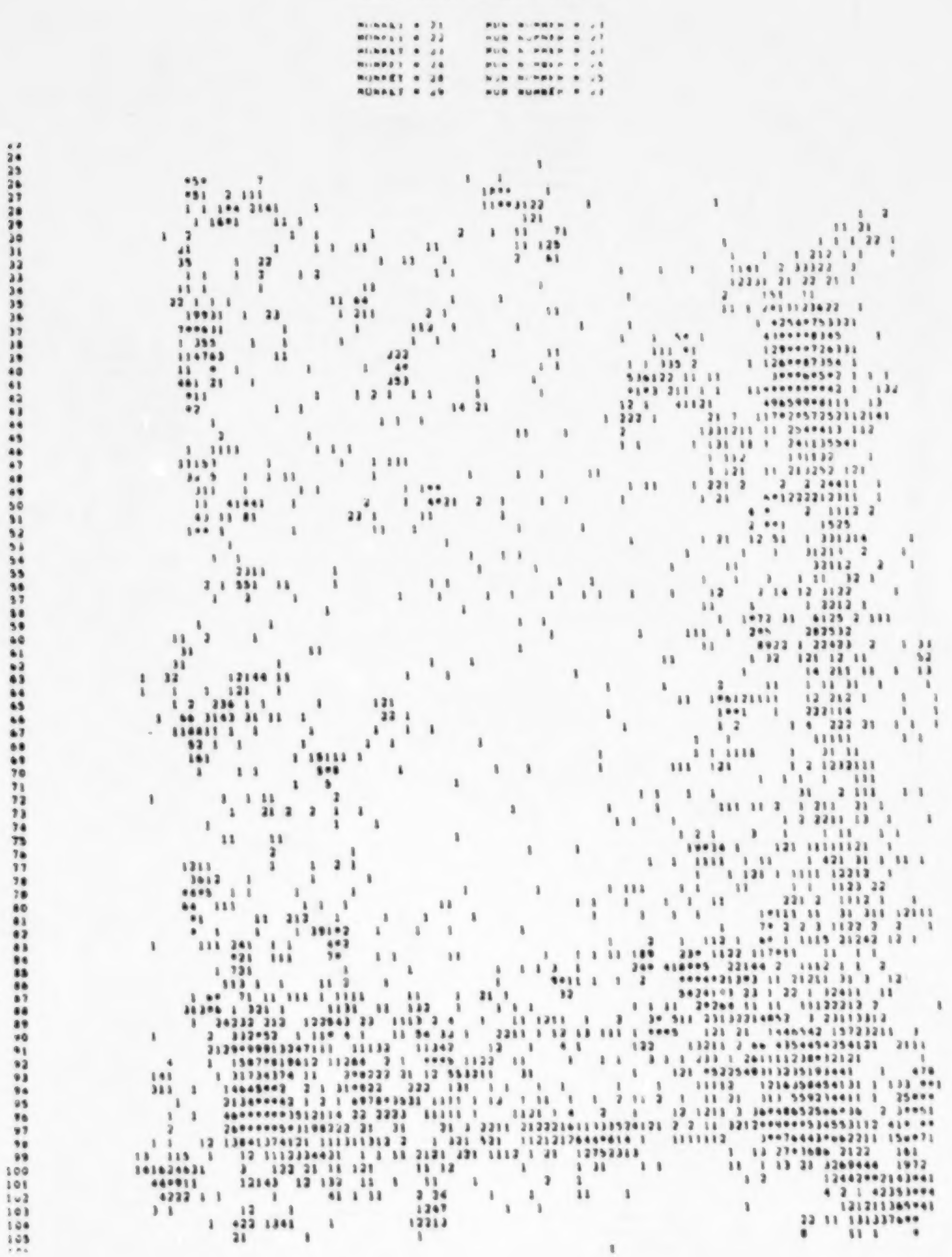

Figure 1. Location of primates in the observation cage as seen from above.

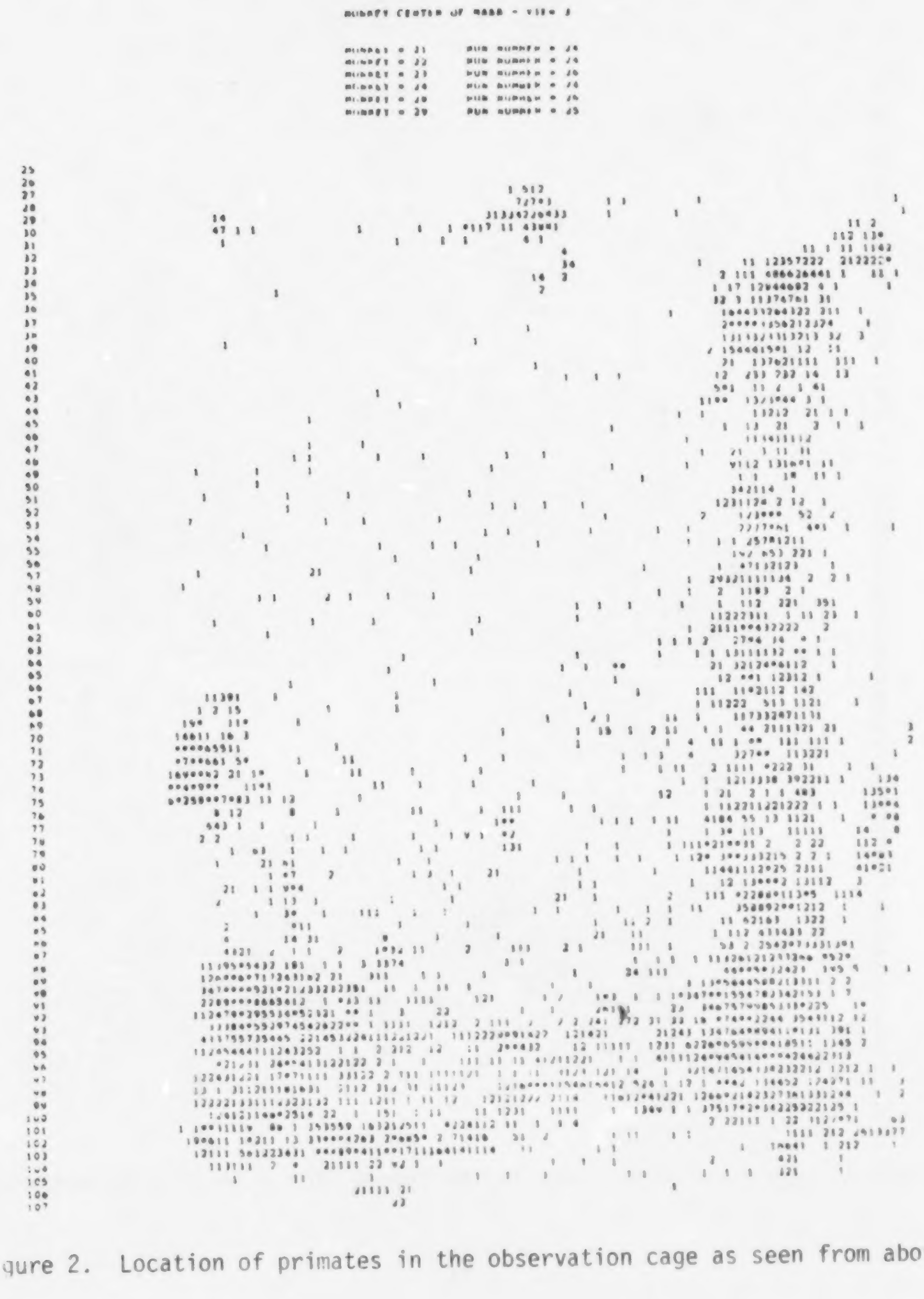

Figure 2. Location of primates in the observation cage as seen from above.

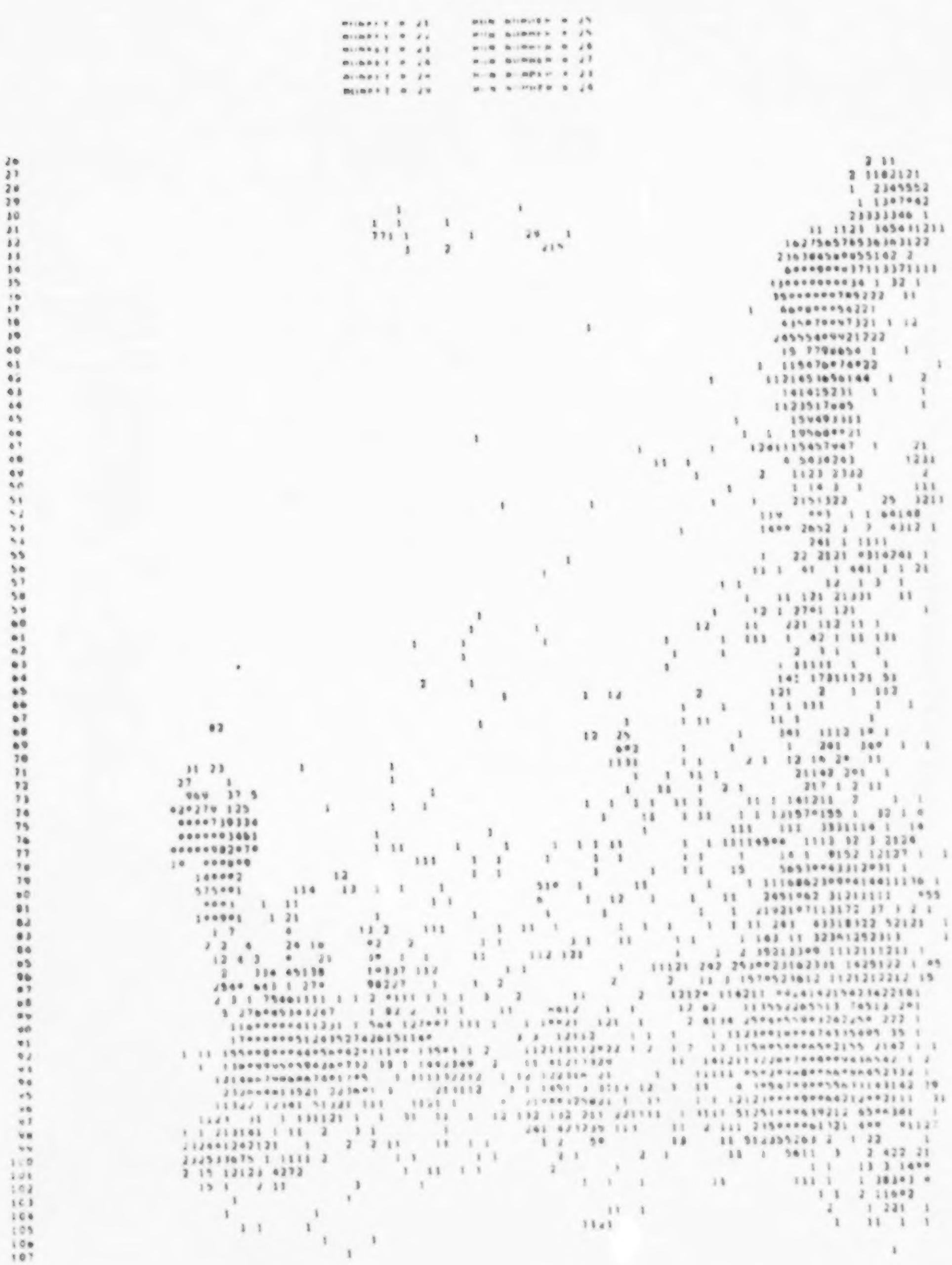

Figure 3. Location of primates in the observation cage as seen from above.

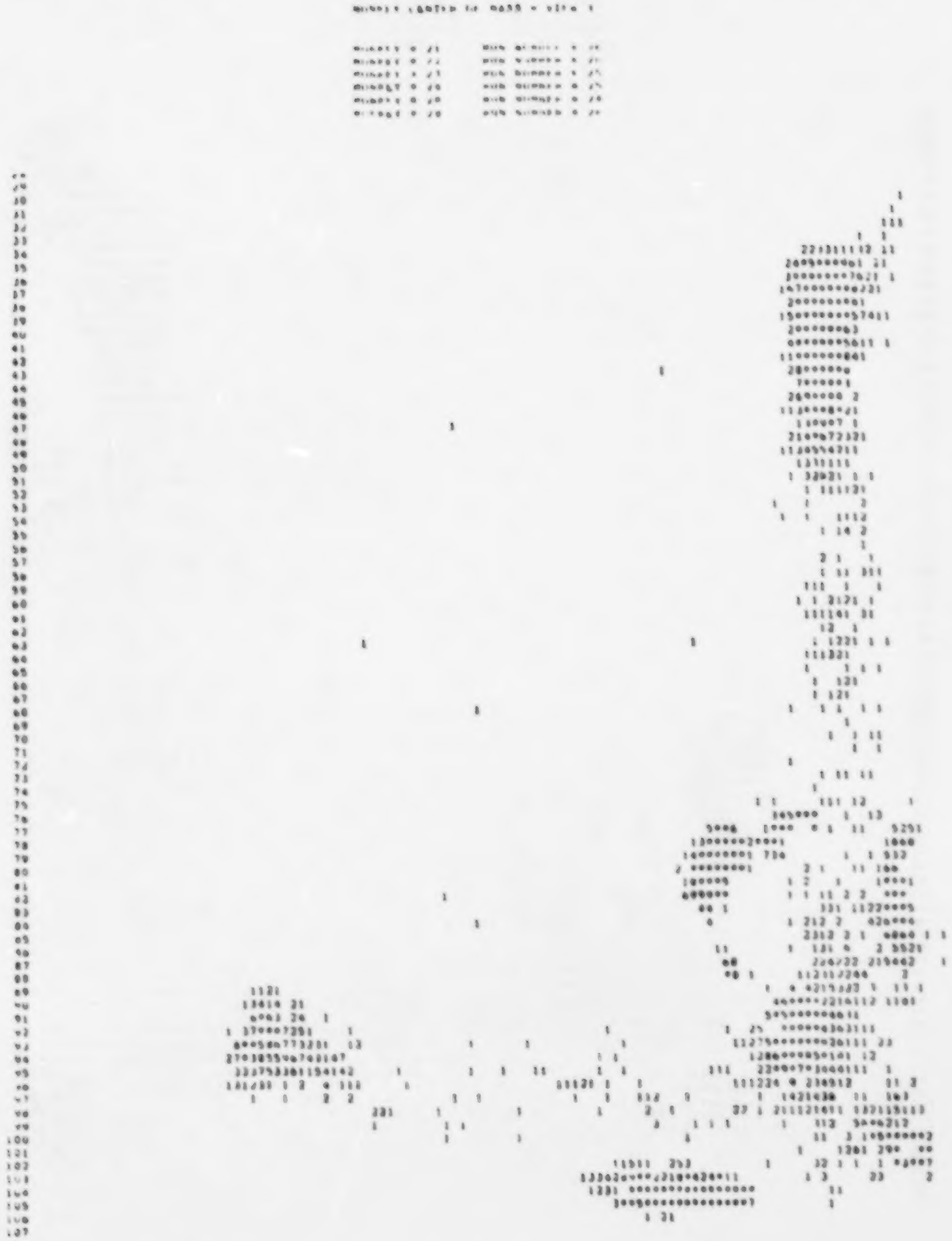

Figure 4. Location of primates in the observation cage as seen from above.

TABLE 1 DELAYED RESPONSE PARADIGM
DEXTRO-AMPHETAMINE - NUMBER OF CORRECT RESPONSES

		Replication 1				Replication 2			
	Monkey	Control	Low	Middle	High	Control	Low	Middle	High
Males	3	38	11	18	0	36	42	42	0
	4	40	39	0	0	36	41	0	0
	5	41	38	0	0	34	38	0	0
	11	33	0	0	0	25	31	0	0
	12	42	38	23	0	41	39	0	0
	30	38	31	21	0	38	35	33	0
Females	18	40	40	0	0	37	21	10	0
	25	9	30	0	0	29	33	0	0
	26	23	36	25	0	36	35	13	0
	$\overline{X}$	33.8	29.2	9.7	0	35.0	35.0	10.8	0

Control = (saline i.m
Low = 0.11 mg/kg d-amphetamine i.m.
Middle = 0.33 mg/kg d-amphetamine i.m.
High = 1.00 mg/kg d-amphetamine i.m.

sensitive detectors of behavioral changes which might be produced by neurotoxicants mobilized into the environment through energy related sources. To test this hypothesis, tests on primates exposed to thallium and methylmercury (two trace elements found in coal) will begin in the near future. It is anticipated that relatively low exposure to these neurotoxic agents will result in significant alterations in the behavior of the exposed primates.

A final point, the functional capability of the central nervous system is an important index of toxic effects, and functional alterations measured as behavioral changes can and do often occur in the absence of a measureable clinical effect. The procedures developed and tested under these tasks should provide needed information concerning the effects of a number of environmental neurotoxicants on the functional capability of the central nervous system.

Bibliography

Since the work for these tasks is currently being conducted, manuscripts are presently being prepared for publication.

Related Research

Lead-Induced Behavioral Changes in the Neonatal Primate - Contract 68-03-2524. Negotiated Contract Branch, Environmental Protection Agency, Cincinnati, Ohio. Four year project, funded for $433,661.00.

The scope of this research is to obtain a controlled, systematic picture of the behavioral and physiopathologic effects of prenatal and early postnatal lead exposure in the non-human primate. The specific objectives of this task are to: (1) assess the effects of *in utero* and early postnatal lead exposure on the central nervous system of the non-human primate as measured through tests of sensorimotor coordination, spontaneous activity and operant learning behavior, (2) evaluate the feasibility of catecholamine turnover, and the potential paradoxical reactions of amphetamine and phenobarbitol in lead exposed primates, and (3) to determine the suitability of the cynomolgus monkey as a primate model for studying the probable neurologic and physiologic effects of environmental lead exposure to humans.

H. TASK TITLE: Detection of Genotoxic Effects of Environmental Chemicals in Cultured Liver Cells

HERL/RTP TASK NO:	8197
CONTRACTOR:	American Health Foundation
CONTRACT NO:	68-02-2483

Summary

Several *in vitro* liver culture systems have been developed and are being validated and implemented to bioassay environmental chemicals for

genotoxic effects. This contractor has developed the following two systems.

A. Induction of autoradiographic unscheduled DNA synthesis (DNA repair) in primary nonreplicating liver cell cultures. This system has been validated with 27 chemicals from 5 chemical classes requiring metabolic activation.

B. Mammalian liver cell mutation assay using continuous epithelial cultures derived from rat liver.

Scope and Objectives

The main objective of this proposal is to develop and validate *in vitro* liver cell culture systems for assessing the genotoxic effects of chemicals and subsequently to apply those systems to identification of environmental effluents possessing such hazardous properties.

Specific objectives of this research are:

A. To ascertain and maximize the sensitivity of primary nonproliferating rat liver cell cultures as a bioassay system responding to genotoxic agents as measured by DNA repair.

B. To ascertain the potential of long-term proliferating rat liver cell cultures to respond to genotoxic agents as measured by DNA breakage, chromosomal damage, mutagenesis, and neoplastic transformation.

C. In validated systems, to test environmental agents.

Background and Approach

The consequences of the interaction of chemicals with genetic material, i.e., DNA, include toxic, lethal and heritabel effects which have been referred to by the general expression "genotoxic". Genotoxic effects may, thus, result in chronic disease through damage to somatic cells, in mutations and birth defects through heritable genetic effects on germinal cells, and in cancer, perhaps as a result of interaction with genetic material in somatic cells. Therefore, the genotoxic effects of environmental chemicals require careful surveillance.

The agents which are expected to pose the greatest hazard to humans are those which are stable, i.e. non-reactive in the environment, but are metabolically activated by an exposed organism. Thus, systems for screening for genotoxic agents must possess broad capability for metabolizing chemicals. Recent review of the metabolism of chemical carcinogens has revealed that liver possesses all the enzyme systems involved in metabolism of carcinogens known to require enzymic activation. Also, liver has been recognized as the best general enzyme source for activating chemicals to their mutagenic metabolites. These facts indicate that liver cells are the cells of choice for obtaining *in vitro* systems with broad metabolic capabilities.

In a metabolically active cell culture system, the genotoxic effects of chemicals can be detected by, at least, five end points as follows: the induction of DNA repair in primary cultures as evidence of DNA damage; the production of DNA or chromosomal damage in long-term cultures; the induction of mutations in long-term cultures; and the induction of transformation in long-term cultures. Thus, the present proposal concerns the development of rat liver cell culture systems for quantitative assessment of genotoxic effects of chemicals.

The objectives of this contract will be pursued by studies involving the following two rat liver cell culture systems: (A) short-term primary nonreplicating cultures and (B) long-term continuous epithelial cultures. The ability of carcinogens to elicit DNA repair will be measured by the induction of autoradiographic unscheduled DNA synthesis in primary cultures. This work will focus on validation of the system and means of enhancing sensitivity such as modification of treatment conditions, preinduction of drug-metabolizing enzymes, and utilization of additional species. The ability of the cells to metabolically activate procarcinogens will also be evaluated by study of parameters of drug metabolizing enzyme systems such as cytochrome P450 levels and benzo(a)pyrene metabolism. In addition, scintillation counting of DNA repair incorporation will be examined as a possible simplification of the assay. The mutagenicity of carcinogens will be studied in the continuous cultures using induction of 8-azaguanine and 6-thioguanine resistance. Efforts will be made to develop additional markers such as thymidine kinase deficiency. Upon satisfactory development of the assays, validation will be performed and testing of environmental samples undertaken.

Research Accomplished

The following two assays have been implemented:

A. Unscheduled DNA repair in primary liver cells (hepatocytes).

B. Liver cell mutagenesis assay in continuous rat liver cell lines.

The DNA repair assay in primary cultures of rat liver cells has been implemented and validated for five classes of chemicals by testing a total of 27 chemicals from the following classes: aromatic amines, aminoazo dyes, mycotoxins, polycyclic aromatic hydrocarbons and nitrosamines. In each chemical class, chemicals were selected based on the available whole animal carcinogenicity data so that positive, weakly positive and negative compounds, where possible, were tested in each chemical class. Most of the chemicals tested required metabolic activation to produce the reactive (mutagenic or carcinogenic) species. The metabolic activation was accomplished without an added activation system, indicating that these primary liver cells are capable of activating a variety of pro-carcinogens or pro-mutagens of different chemical class. The 15 positive carcinogens were all positive in the DNA repair assay. Of the three weakly positive carcinogens tested, two were positive in the DNA repair and one, benzanthracene, was negative. All nine structurally related noncarcinogens were inactive, except 4-acetylaminofluorene, which is mutagenic in Ames *Salmonella typhimurium* metagenesis assay.

In the mutagenesis assay the mutants have been characterized and the optimal expression time determined.

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Williams, G. M. In Press. Further Improvements in the Hapatocyte Primary Culture DNA repair Test for Carcinogens: Detection of carcinogenic biphenyl derivatives. Cancer Letters.

Related Research

We are evaluating a variety of in vitro test systems to screen environmental chemicals for potential carcinogenic and mutagenic activity. Most of these test systems require the addition of an exogenous metabolic activation system. The liver cell systems offer the potential advantage of an internal activation system.

I. TASK TITLE:

Biological Assessment of Exposure to Sulfur Dioxide and Acid Sulfate

HERL/RPT TASK NO: 8187

CONTRACTOR: Duke University

CONTRACT NO: R0805622-01

Summary

Because the aim of this project is to explore the possibility of devising a biological test of human SO_2 exposure, assay procedures for detection of S-sulfonates are being developed. These procedures will then be applied to experimental animals for quantitating SO_2 exposure.

Scope and Objectives

Investigations of the killer smogs of London, Donora and the Meuse Valley together with the mortality studies of Schimmel and colleagues in the 1960's and the C.H.E.S.S. studies of the late 1960's and early 1970's have all implicated atmospheric SO_2 as a danger to public health. Controls of SO_2 emissions have been put in force as a result of this data. These controls have essentially banned the use of cheaper fossil fuels in metropolitan areas. A severe problem with all these morbidity and mortality correlation studies is the tenuous connection between the ambient air concentrations of SO_2 as measured at fixed air quality monitoring stations and the SO_2 levels to which the affected individuals are actually exposed. Not only do ambient levels vary considerably by location within the same city (Goldstein), but those who seem most affected, individuals with pre-existing cardiorespiratory impairments, are predominantly located indoors.

The use of personal air samplers in the large studies needed to examine the low levels now being discussed in relation to air pollutants have obvious, severe drawbacks. Earlier work (Gunnison; Irreverre et al.) has shown that absorbed and endogenous SO_2 results in the formation of free and protein-bound sulfonated ($-S-SO_3$) cysteine both in the blood and tissues. If a sufficiently sensitive assay for these sulfonates could be developed, it may be possible to correlated cumulative exposure to SO_2 to the S-sulfonate levels in single blood samples. It is to that end that we are working.

Such an assay could also be used in monitoring occupational exposures. Workers in pulp mills, metal ore smelters, acid plants and lime plants are exposed to higher and even more variable levels of sulfites than general urban populations. A simple blood test for cumulative exposure might be useful in these settings for both research and routine monitoring.

Background and Approach

It has previously been shown that sulfite injected in vivo (Gunnison, 1971) and SO_2 inhaled by humans (Gunnison, 1974) results in a proportionate rise in plasma S-sulfonates. An assay for these sulfonates has been developed (Gunnison, 1973), but in human exposure experiments it was found that the precision of the method in the 1-10 ppm range of plasma S-sulfonates was no better than ± 25%. The human data (Gunnison, 1974) showed that approximately a change of 1.1 nanomoles per ml (roughly 0.1 ppm) of plasma S-sulfonate resulted from each 1.0 ppm increment in SO_2 exposure. Obviously this assay is not adequate for environmental monitoring where the present air quality standard requires levels to be held to the ppb range.

In order that our assay be as cheap, reliable and simple to perform as possible, we have chosen to release the sulfite from all the plasma S-sulfonate at one time so that one assay for sulfite would be all that is necessary. We accomplish this by adding cyanide in large excess and at high pH. The cyanide displaces the sulfite from all the sulfonated -SH groups all at one time (Gunnison, 1973; Nor and Tabatabai).

Sulfite Assays

We began the project by conducting an extensive search of the environmental, occupational health, analytical chemistry and toxicology literature for sulfite assays of sufficient sensitivity. Several promising methods were found and are listed below with brief descriptions.

1. West and Gaeke Method as Modified by Gunnison

   This is a colorimetric technique using acid bleached pararosaniline. Its lack of sensitivity and precision at levels of interest is mentioned above.

2. Ellman Reagent (Johnston, Murray and Cain)

   This assay was developed to follow the production of sulfite in bacterial colonies. It, like the pararosaniline method, is interfered with by thiols and probably cyanide also, but the authors describe techniques by which they claim the interference can be eliminated. Sensitivity is claimed to be in the 0.1 micromole per ml level.

3. Polarography

   Aulenbach and Balmat have used a simple D.C. polarographic technique for the detection of sulfite in sewage sludge down to the 1.1 ppm level. Brinkman Instruments has technical papers available describing a simple and apparently commonly used method in the paper and pulp industry for determinations below 1.0 ppm.

4. Radioactive Labeled Adducts

   S. Harvey Mudd developed a very sensitive method for urine and tissue extracts using ^{14}C-N-ethylmaleimide in conjunction with his studies of a child with a congenital deficiency of sulfite oxidase. Nakamura and Tamuta have used both N-ethylmaleimide (NEM) and p-aminobenzoic acid (PABA) to adduct with $^{35}SO_3^=$ to assay sulfite released by a bacterium. Labeled PABA is not as readily available from commercial suppliers is NEM, however.

5. Sulfite Oxidase

   This is an enzyme that our laboratory has much experience with. It catalyzes the oxidation of sulfite to sulfate and can use oxidized cytochrome c as an electron acceptor. By following the production of reduced cytochrome c spectrophotometrically the activity of the enzyme or the concentration of sulfite can be quantitated.

Research Accomplished

Most previous workers have used the Segel and Johnson or the Clark method to produce S-sulfocysteine. Both of these methods produce a sulfonate heavily contaminated with cupric ions. While the cupric ion is used to catalyze the formation of the sulfonate it also catalyzes its degradation (Sorbo). Since plasma has little unbound copper present, we need a product free of catalyst to be sure that our cyanolysis of the synthetic sulfonate is applicable to the conditions found in plasma. Inglis and Liu developed a method of synthesis without using any cupric catalyst. We have successfully repeated their procedure and have produced basically Cu^{+2}-free S-sulfocysteine.

It has been our ultimate goal to develop several assays from the above possibilities and then compare them "head-to-head" on identical samples to determine which offers the most in sensitivity and precision for the least cost in time and money. We have been able to perform the pararosaniline assay (Gunnison, 1973) with sensitivity comparable to that reported.

An assay for sulfite in plasma using sulfite oxidase has been developed. It is sensitive down to a level of 1.0 nanomole per ml. A severe problem arose when attempting to use the enzyme to assay for S-sulfonate however. Cyanide reacts readily with cytochrome c producing a prohibitve amount of interference even in a dual-beam spectrophotometer. At the moment the enzyme offers a sensitive method for assaying sulfite in plasma, but as yet is not applicable for measuring levels of sulfonate.

Much to our surprise the differential pulse polarogram of out-dated human, blood bank plasma turned out to be flat in the region of the anodic wave of sulfite. After acidification, added sulfite produces a measurable wave even down to nanomoles/ml levels. The technique is so sensitive, in fact, that every unit of out-dated blood we have looked at shows a sulfite wave after incubation with alkaline cyanide. Presently our efforts are directed at producing sulfonate-free plasma so that we may run a standard curve in human plasma. A standard curve run in normal saline showed sensitivity and reproducibility down to micromolar sulfite concentrations. While the need for absolute deoxygenation makes the polarographic technique slow, there is a machine available from Princeton Applied Research which could automatically perform the assay. Thus this technique could still be quite useful for large scale screening programs.

Bibliography

None.

Related Research

In epidemiological studies on SO_2 toxicity it is essential to recognize the fact that sulfite oxidase plays a role in the detoxification of SO_2 by oxidizing it to sulfate. Since the activity of the enzyme is totally dependent on the presence of molybdenum, tissue levels of the enzyme would be governed by the molybdenum nutritional status of human populations. Population studies on sulfite oxidase levels would, therefore, be of great use in this connection. We have found serum and various blood constituents do not contain sulfite oxidase activity in detectable levels, making this readily available source useless for such studies. We have, however, found that human fat tissue, also used as a source by epidemiologists, does contain sulfite oxidase. We are also testing the possibility that sulfite oxidase activity could be induced in activated human lymphocytes.

J. TASK TITLE:

In Vivo Methods for Assessing Neurotoxicity

HERL/RTP TASK NO: 8185

GRANTEE: U. of Cincinnati

GRANT NO: R80569310

Background and Approach

Because of increasing concern over the growing number of potentially neurotoxic agents which are entering our environment, there has been an increased emphasis on investigations pertaining to the neurochemical, neurophysiological and behavioral effects which arise as a result of exposure to such neurotoxins. In many cases this research has been characterized by a high degree of empirical data collection with little or no attempt to formulate unifying principles which allow us to extrapolate beyond the original data. One of the primary reasons for this is the failure to employ research strategies which employ hypothesis testing and the development and modification of conceptual models of the systems under consideration.

An additional problem has been our inability to extrapolate between various levels of analysis. In order to facilitate the translation of observations at one level of analysis, e.d. behavior, to another level of analysis, e.g. biochemical mechanisms responsible for the behavioral changes, one needs a test system which incorporates the following characteristics:

1. In order to minimize the cost, both in money and time, necessary to carry out an investigation, the procedures should be simple. This rules out behaviors which require weeks of elaborate training, or relatively sophisticated surgical manipulations such as precise lesioning or the chronic implantation of cannula or electrodes.
2. The behavior should be a discrete "unit of behavior" mediated at the central level (since this is the target organ of concern) but analogous to the discrete sensory-motor reflect arc seen at the spinal level; i.e., stereotyped and easily and reproducibly elicited.
3. A "unit of behavior" for which the neuronal circuitry is fairly well known. This of necessity tends to restrict the selection of behaviors to those tied fairly closely to the motor end of the sensory-motor chain. At higher levels of sensory-motor integration the complexity is such that our understanding of neuronal circuitry is extremely sketchy.
4. A method must be available for the delivery of the toxic agent to the intended test system without disrupting the function of other systems which might interact indirectly with the system under study.
5. In order to facilitate hypothesis testing, it is necessary that the behavior to subject to environmental and pharmacological manipulations which would lead to predictions of the nature of the biochemical lesion prior to biochemical determinations.

> Unless the system can be manipulated in such a manner, we will be left with nothing but the concurrent observation of one behavioral change and one biochemical change and no means of demonstrating a cause-effect relationship. While the collection of empirical observations at all levels of analysis is necessary to provide an adequate data bank, we must move beyond this approach and begin hypothesis testing.

There are several possible behaviors mediated by the extrapyramidal system, e.g. tremor or rotational behaviors, which meet these criteria and might be developed into useful test systems for the study of neurotoxins. Of these two, rotational behavior appears to be the most promising. This behavior provides a way to study synaptic function as it is reflected in a simple, quantified behavior that is empirically linked to dopamine transmission.

Studies to be proposed here will be restricted to an investigation of the neurotoxic effects of inorganic metals including lead, cadmium, mercury, manganese, copper and zinc. The metal to be most extensively examined will be lead. The reasons for this selection are as follows: (1) Heavy reliance on fossil fuels will result in the introduction of numerous metals into our immediate environment. These include: lead, manganese, arsenic, fluoride, nickle, cadmium, zinc, and copper. (2) Past experience tells us that lead ranks very high on any list of those agents which constitute a significant health hazard to a significant segment of our population. (3) The behavioral and biochemical effects of low level chronic exposure to lead have been investigated extensively by this applicant for the last two and one-half years, and (4) data from several laboratories suggest that chronic exposure to inorganic lead produces alterations in both the catecholamine and acetylcholine systems. Since these systems are also intimately involved in the mediation of rotational behavior, this test system should be sensitive to the neurotoxic effects of lead. Cadmium and mercury will also be examined in later studies in order to determine if a common mechanism of action can be established to account for the neurotoxicity of this group of heavy metals. The essential trace metals, manganese, copper and zinc may be evaluated in interaction studies with the heavy metals to determine if it is possible to alter the neurotoxic actions of the heavy metals.

Scope and Objectives

The primary objective of this proposed research effort is to develop a methodology for the *in vivo* testing of known and/or potential neurotoxic agents. This approach will rely heavily upon a procedure, developed by Anden *et al*. in which the functioning of the extrapyramidal system is reflected in an animal's asymmetric posture and locomotion. This "rotational behavior" will be used (1) to evaluate the effects of acute intracerebral administration of neurotoxins and (2) to unmask covert changes in neural function which may arise as a result of long-term low level systemic exposure to neurotoxins. The aim is to develop an approach which lies between strict behavioral analyses of neurotoxicity which do not lend themselves to studies of mechanism of action, and the more classical *in vitro* biochemical and neurophysiological assays which do not readily permit extrapolation to the behavioral level. Initial studies will focus on the usefulness of this methodology in the assessment of neurotoxicity arising from exposure to heavy metals, particularly inorganic lead, cadmium and mercury.

Objectives --

The specific objectives are designed to provide information relevant to the following general hypothesis: rotational behavior provides a sensitive test system for the evaluation of the extent and mode of action of neurotoxins. Furthermore, the test system is well suited to the formulation and testing of hypotheses pertaining to the neurochemical site of action of neurotoxins. To explore this hypothesis, the following specific aims are proposed:

1. Develop a homogenous test population of rotating C57Bl/6J mice by the technique of intrastriatal injection of 6-OHDA.
2. Define the pharmacological effects and biochemical extent of such lesions.
3. Evaluate the sensitivity of this behavioral test system to the influence of intrastriatally injected lead or chronically ingested lead.
4. Evaluate the usefulness of this test system in predicting the presence of biochemical changes following exposure to lead.
5. Compare the sensitivity of this test system with more commonly used behavior test systems such as locomotor activity or learning studies.
6. Examine cadmium and mercury toxicity with the rotational test system. Compare findings with those obtained with lead in order to detect any common mode of action.

Expected Benefits

Over the years there has been a steady increase in the number of potentially neurotoxic agents being introduced into the environment, e.g. heavy metals, pesticides and solvents. Before appropriate and reasonable controls can be instituted, it is necessary to identify which agents represent a threat. This is no small task when one considers the number of agents to be tested, the chemical diversity of the agents to be tested and the complexity of the target system. There is need for a test system for screening purposes comparable to those being developed in the field of carcinogenesis and mutagenesis. The usefulness of such a screening test could be greatly enhanced if the test system also permitted the study of mechanism of action.

The rotational model described in this proposal could fulfill the need for a relatively rapid *in vivo* screening test. In addition, if developed to its full potential, the test system should permit hypothesis testing, which is necessary for identifying the site and mechanism underlying the observed behavioral toxicity. It may eventually permit the development of unifying concepts based on common mechanisms of action. For example, the following questions can be asked: Do all heavy metals (or solvents or pesticides) produce the same pattern of effects on rotational behavior? Do all demyelinating agents produce the same pattern? Do anions produce uniquely different effects than cations? These questions have been asked before, but not in an *in vivo* behavioral test system which permits this degree of experimental control.

In addition, the test system may permit the evaluation of potential therapeutic approaches, e.g. displacement of heavy metals by essential trace metals or the development and testing of new chelating agents with special reference to the CNS.

Obviously not all neurotoxins have the striatum as their principal site of action. However, this region is neurochemically representative of the neural elements and supportive tissue which are usually regarded as neurotoxic target tissue. Evaluation of the neurotoxicity observed in this region will suggest what to look for in other brain regions when the agent is administered systemically.

The test system is being used as a model system for testing drugs used to treat Huntington's disease and Parkinson's disease. Hopefully it will be at least as useful in identifying and studying environmental agents which have neurotoxic potential.

SECTION 3

DETERMINE THE METABOLISM AND FATE OF HAZARDOUS AGENTS ASSOCIATED WITH ENERGY TECHNOLOGIES

A. TASK TITLE:

The Effect of Whole Animal Exposure to Acid Mists and Particulates on the Pulmonary Metabolism of Benzo(a)pyrene in the Isolated Perfused Lung Model

HERL/RTP TASK NO: 8147

CONTRACTOR: University of Cincinnati

CONTRACT NO: 68-02-1678

Summary

The isolated lung perfusion (IPL) technique was used to study in an "in vivo" situation, the effects of SO$_2$, crude air particulate (CAP), and benzo(a)pyrene (B[a]P) treatment on benzo(a)pyrene metabolism. The influences of these agents on the metabolism of benzo(a)pyrene, an environmental carcinogen, may explain differences in the carcinogenicity of this agent. S)$_2$, CAP and B[a]P pretreatment increased B[a]P metabolism in the IPL and increased the formation of specific activated metabolites indicating that these agents may have cocarcinogenic activities.

Scope and Objectives

The long term goal of this research is to assess the effects of environmental contaminants on the pulmonary metabolism and distribution of the carcinogen benzo(a)pyrene. This research attempts to clarify whether a change in metabolic rate, metabolic pathway or distribution in the tissues could account for differences in the carcinogenic response.

An isolated perfused lung preparation, developed previously by the investigators of this contract, is being used for this study. Various combinations of crude air particulates, microsomal enzyme inducers, and acid mists are used as a pretreatment regimen before the intratracheal administration of ^{14}C-benzo(a)pyrene (alone or in combination with crude air particulate). Blood taken at various times throughout the perfusion and tissues are extracted with organic solvents and concentrated. The metabolites are chromatographed and quantitated using liquid scintillation counting.

The metabolites of B[a]P, their rates of formation, and their distribution in various tissues are determined.

Background and Approach

Inhalation has been the main mode of exposure of humans to agents known to be casually associated with an increased incidence of respiratory cancer. Epidemiological and experimental evidence indicates that the interplay of multiple environmental factors is responsible for the induction of lung cancer. Man is exposed to a complex mixture of potentially hazardous materials including specific carcinogens and a variety of agents which may modify the manner in which the lung disposes of inhaled materials. It is well established that the lungs are capable of binding and metabolizing such agents. One such carcinogen is benzo(a)pyrene, a ubiquitous environmental pollutant formed during the destructive distillation of coal and in other processes involving incomplete combustion of organic materials. B[a]P occurs as both a common contaminant of the urban environment and as a constituent of tobacco smoke. Its metabolites exhibit varying degrees of mutagenicity, carcinogenicity and toxicity.

A major requirement for understanding the mechanism of B[a]P carcinogenesis is a detailed knowledge of the rate and pattern of formation of metabolites, and the factors controlling their formation. Such factors include particulate matter which carries a multitude of chemicals, including B[a]P, which may be deposited in various regions of the respiratory tract. The ambient air of both occupational and urban settings contain many such small particles. It has been established experimentally that B[a]P in combination with ferric oxide produces tumors of bronchogenic origin with an incidence of up to 100%. Carbon particles with B[a]P also produces a high incidence of lung tumors. The particulate effect has been suggested as a means of providing longer residence times at the target tissue, but the biochemical effect has not been fully investigated.

There is, however, no way to study the pulmonary metabolism of B[a]P *in vivo* because of the metabolic influence of other organs. *In vitro* tissue preparations, such as slices and homogenates, are not satisfactory for studies involving concurrent administration of multiple agents in different physical forms, distribution determinations or binding of compounds throughout the pulmonary system. Therefore, the isolated perfused lung (IPL) appears to be the best *in vivo* preparation for investigating pulmonary metabolism of foreign compounds especially compounds adsorbed onto particulate. An important aspect of current work is the assessment of the rate of formation and types of metabolites formed when B[a]P is administered with ferric oxide or crude air particulate (CAP) on the IPL.

Research Accomplished

SO_2 Inhalation - So far, rather startling and surprising results have been obtained using low concentrations of SO_2 (1-2 ppm) in two animals.

When compared to control (no pretreatment), the inhalation of 1-2 ppm SO_2 *in vitro* results in (1) a large increase in the total rate of formation of the metabolites of B[a]P and (2) a change in the distribution of the metabolites. There also appears to be a lot more nonextractable material in the SO_2 experiments than the control. This is all the more interesting because there is no pretreatment of the SO_2 group.

This very large rate of formation of metabolites in the SO_2 experiments is similar to B[a]P pretreatment. However, the distribution is quite different. There is more 7,8-dihydrodiol and less 9,10-dihydrodiol, diones and monohydroxylated metabolites in the SO_2 experiments than the B[a]P pretreatment experiments. This indicates that SO_2, in conjunction with B[a]P, affects the metabolic pathway in ways which are different from the B[a]P pretreatment or control (no pretreatment).

CAP Treatment--

(1) Crude air particulate (CAP), when administered with ^{14}C-B[a]P to the IPL, decreased the total metabolic rate in comparison to the appropriate control.

(2) CAP administered intratracheally (IT) increases the total metabolic rate of B[a]P in the IPL in comparison to the appropriate control.

(3) CAP, when administered IT either as a pretreatment regimen or *in vitro* to the IPL, causes an increase of the 7,8 and 9,10-dihydrodiols and a slight decrease of the monohydroxylated and dione metabolites when compared to the appropriate control.

(4) CAP when administered IT either as a pretreatment regimen or *in vitro* to the IPL causes a decrease in nonextractable material except when B[a]P is given as a pretreatment.

All of these conclusions indicate that the CAP affects the metabolite pathway of B[a]P such that more 7,8 and 9,10-diol are present. CAP also affects the rates of formation of the metabolites depending on the treatments. These are at present only preliminary conclusions. Statistical analyses are being done on the data using various model systems.

B[a]P Treatment--

Both B[a]P administered intraperitoneally (IP) and IT pretreatment increase the metabolic rate of B[a]P administered IT to the IPL preparation. A change of the relative percentages of the metabolites, especially the 9,10-dihydrodiol, is evident in both pretreatment groups compared to the control. The IP B[a]P pretreatment increased rate versus IT B[a]P can be accounted for by the corn oil administration. Both IP and It B[a]P pretreatment and 3-methylcholznthrene stimulate 9,10-dihydrodiol, whereas the corn oil increases the 7,8-dihydrodiol and the nonextractable metabolites.

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Related Research

None.

B. TASK TITLE:

Evaluate Influence of Inhalation of Acid Aerosols, H_2SO_4, SO_3, HNO_3 and Particulates on Production of Chronic Lung Disease in Rats, Guinea Pigs and Primates.

HERL/RPT TASK NO.:	8148
CONTRACTOR:	University of California at Davis
CONTRACT NO.:	68-01-1721

Scope and Objective

The objective of this project is to evaluate the nature and extent of functional and morphological dose-related responses of the respiratory system resulting from inhalation of small droplet H_2SO_4 aerosols as a function of mass concentration, droplet size, duration of exposure, and duration of post-exposure period. Initially, rats and subsequently guinea pigs, and nonhuman primates will be studied. Physiology (pulmonary function), morphology (transmission electron microscopy, scanning electron microscopy, and histochemistry), biochemistry (glycoprotein synthesis), and pulmonary clearance will be evaulated in control and exposed groups.

Research Accomplished

The exposure facility has been developed and four groups of 100 rats each are being exposed 24 hrs/day for 180 days to the following atmospheres: (1) submicron H_2SO_4 mist (0.05 μM, CMD) at 1 mg/m^3; (2) ozone, 0.5 ppm; (3) H_2SO_4 plus ozone; and (4) control air. These animals will be studied at 90 and 180 days for morphological changes (using SEM, TEM, and histochemistry for mycopolysaccarides), biochemical changes (rate of glycoprotein synthesis by in vitro systems) and physiological changes (pulmonary function tests of lung volumes and mechanics). New methods of increased sensitivity have been developed to determine mucopolysaccaride histochemically.

Related Research

This project is supplementary to on-going in-house research into the health effects of these environmental pollutants. The EPA in-house research primarily deals with ultrafine H_2SO_4 singly and as produced by a complex reaction with SO_2, O_3 and a hydrocarbon.

C. TASK TITLE:

Determination of the Effects of Material from Alternate Energy Sources on Upper Respiratory Tract Clearance Mechanisms.

HERL/RTP TASK NO: 8149

CONTRACTOR: Dr. Dorothy Adalis

CONTRACT NO: 68-02-2295

Summary

The investigator will test the cytotoxicity of a number of environmental chemical substances employing an in vitro model that is designed to measure subtle changes in upper respiratory tract clearance mechanisms. The following bio-indicators of effect will be measured; ciliary frequency activity, morphological and histological alterations, and biochemical changes. Dose-response studies will be conducted using various concentrations and length of exposure.

Scope and Objective

In general, the objective of this research project is to screen a variety of chemical substances in order to determine their potential toxic effect on mucociliary activity. An in vitro model using isolated tracheal rings of the hamster will be employed to determine significant adverse effects on ciliary frequency activity, morphological and structural changes and some biochemical assay of the functional state of these isolated cells.

Samples to be tested may include materials derived from the shale oil, coal gasification and liquefaction plants and particulate effluents from both mobile and stationary sources. Additional substances, both soluble and insoluble may also require testing. These substances shall include, but not be limited to, metallic substances found in the atmosphere.

The contractor shall test the cytotoxicity of a variety of substances on ciliary activity. Several concentrations will be tested in order to determine the dose-response relationship.

Background and Approach

It is expected that a number of new substances will be emitted into the environment when new alternate sources of energy are developed and utilized. Many of these substances which might be released during the utilization process have not been toxicologically evaluated for their potential health effects. Thus, it becomes necessary to screen a number of these substances for their detrimental effect on the upper respiratory clearance. The material expected to be tested may include, but not be limited to, substances derived from shale oil, coal gasification and liquefaction plants and from particulate effluents from power stations, stationary engines and motors from various mobile sources. These substances could be extremely chemically complex, since they are capable of participating in a wide number of atmospheric reactions, both with particles and with gaseous pollutants. Once these substances are inhaled and deposited within the pulmonary racemus, they may exert a toxic effect on the host via one or more of these mechanisms: (1) the substances may be intrinsically toxic due to their inherent chemical or physical properties; (2) the substances may interfere with one or more of the clearance mechanisms in the respiratory tract; and (3) the substances may act as carriers of an adsorbed toxic substance.

Once deposited upon the tissue, these substances are then brought into intimate contact with very specialized host cells, i.e., cilia and alveolar macrophage, whose function is to rid or clear the body of inhaled substances. If these defense systems of the host are reduced in activity then an accumulation of both viable and nonviable inhaled substances would occur, which in turn would jeopardize the health of the host.

Generally, this study employs an *in vitro* model which will imitate the above occurrence in an organ culture system and allow the investigator to measure the toxicity of a large number of substances on the respiratory cilia of the hamster's trachea. Based on this *in vitro* data, whole animal exposure could then be conducted, which would validate the *in vitro* findings.

The objective shall be to screen a variety of substances for their toxic effect on mucociliary activity using an *in vitro* Hamster Model system to determine significant adverse effects on normal ciliary activity and normal ciliated epithelium. A variety of parameters shall be measured including: ciliary frequency activity, morphological and structural changes and some biochemical assay of the functional state of these isolated cells.

Research Accomplished

In general, the research conducted has followed the original scope of work in that it has evaluated the effects of a number of substances on the upper respiratory clearance mechanism. During this period of performance, the investigator has developed and is using the proposed in vitro animal (hamster) model system for evaluating the cytotoxicity of a variety of substances.

The studies being conducted and planned may be classed as two types: (1) physiological, and (2) histological and cytological. The physiological studies include techniques of ciliary beating frequency determinations, oxygen consumption and ATP content. The histological studies are of two types: microscopic pathology and ultra-structure pathology.

Preliminary studies were conducted to find dose-level responses of a number of pure chemicals that might be found in the effluents of alternate energy sources.

The metals that apparently were non-toxic at 100 µg/ml were molybdenum, barium, lead, chromium (in +3 valance state) sulfite, and magnesium. The metals showing toxicity at 100 g/ml were mercury, zinc, nickel, manganese and cadmium. The most toxic materials in order of increasing toxicity were cobalt, copper and chromium (as chromate). Cobalt required 2 days to show toxicity at 10 µg/ml; copper was lethal in 24 hours at 10 µg/ml and chromium lethal in 24 hours at a concentration of 1 µg/ml.

Samples of material from the electrostatic precipitator of a coalfired power plant, ammonium chloride, and ammonium sulfate were tested for toxicity and, for these samples, concentrations greater than 1 mg/ml were required to cause significant toxicity.

ATP content of rings treated with zinc sulfate, zinc ammonium sulfate or ammonium sulfate were measured. The ATP content of rings treated with $ZnSO_4$ shows a linear dose-response relationship. An even greater decrease was found for $ZnSO_4 \cdot (NH_4)2$ at the same concentration of zinc ion.

Bibliography

The following publications and presentations resulted from support of the program.

(1) Toxic effects of cadmium on ciliary activity using a tracheal ring model system. Environ. Res. 13:111-120, 1977

(2) Cytotoxic effects of Ni on hamster tracheal ring organ culture. Proceed. of International Congress on Toxicology, Toronto, Canada.

(3) Effect of nickel on upper respiratory tract clearance. Submitted to Am. Rev. of Respiratory Disease, 1978.

Related Research

To my knowledge, this investigator is not being funded by any other source to conduct any similar type of research.

D. TASK TITLE:

Comparison of Pulmonary Carcinogenicity of Known Carcinogens With and Without Added H_2SO_4 Mists, Airborne Respirable Particles and Gases

HERL/RTP TASK NO. 8158

CONTRACTOR: New York University Medical Center

CONTRACT NO: 68-02-1750

Summary

This is a four year project which began in 1974. The project began with the development of a primary model for evaluation of pulmonary carcinogenicity of known carcinogens and their relationship to potential co-factors of air pollution. The co-factors being studied are: sulfuric acid mist; other sulfur oxides; airborne particulates; and gases. Exposure is accomplished by first administering carcinogens to Syrian Golden hamsters via intubation followed by inhalation exposure to various co-factors. Initial studies concentrated on benzo(a)pyrene as the primary carcinogen and sulfuric acid mist as the co-factor. Phase I, in 1974, involved range-finding studies of H_2SO_4 mist, which led to the selection of 100 mg/m^3 as the level to be used in combined inhalation-intubation studies.

Phase II, initiated in 1975, includes combined intubation-inhalation studies with benzo(a)pyrene and sulfuric acid mist. Due to the complexity of this phase, the experiment has been segmented into two parts relating to two distinct problems in carcinogenesis: (1) initiation-promotion in the case of single intubation; and (2) co-carcinogenesis in the case of multiple intubations. Each segment has involved 600 hamsters.

Phase III involves the addition of other co-factors alone or in conjunction with sulfuric acid mist.

Phase IV involves the inhalation exposure of animals to the by-products of sulfur dioxide passed through a vanadium pentoxide catalytic converter. Autopsy and histopathology will be performed on all animals. Phase IV required the design and construction of a catalytic reactor for the conversion of sulfur dioxide to sulfur oxides. The reactor consists of a temperature-regulated oven capable of maintaining constant compartmental temperatures, a tubular catalyst bed, and a 13.5 liter reservoir from which samples are periodically drawn for analysis. The conversion efficiency of this catalyst was evaluated. The vanadium pentoxide catalyst was found to be most effective in converting SO_2 to other sulfuric oxides. Animal studies will be designed and performed to characterize the toxicity of the product mixtures produced by the catalytic reactor and aerosol combinations.

Histological examinations have been completed on a limited number of exposed animals. The major lung histology includes early findings of hemorrhage, congestion, and edema, both in single and multiple intubation studies.

A high percentage of tumors in the respiratory tract were found in animals receiving the multiple dose intubations of B[a]P with or without sulfuric acid.

Scope and Objective

The primary objective of this project is to study the response of animals (male golden hamsters) exposed to combinations of environmental contaminants and known carcinogens. Two segments of the research are: (1) initiation-promotion of carcinogenesis as simulated by a single intubation of a carcinogen (benzo(a)pyrene [B[a]P]) followed by lifetime exposure to sulfuric acid mist; and (2) co-carcinogenesis as simulated by multiple intubations of a carcinogen (benzo(a)pyrene) followed by lifetime exposure to sulfuric acid mist. The study will also include response after exposure to B[a]P and metal oxides (vanadium or iron).

Background and Approach

Among the critical problems in air pollution toxicology, the most important appear to be related to the interaction of irritant gases and particulate materials. The frequent occurrence of high humidity and specific particulate materials are conditions which lead to the formation of sulfur oxide intermediates and sulfuric acid aerosols. Among the common atmospheric components which catalyze these transformations are carbon, vanadium oxides, soluble metal salts, and nitrogen oxides.

Examination of the literature indicates the extreme difficulty in assessing the effects of ambient levels of sulfur oxides on human health. The available toxicological data on sulfur oxidation products indicate that sulfuric acid and sulfates are more potent as irritants than sulfur dioxide. This has been demonstrated in studies using mortality and lung pathology as criteria, as well as in studies using alteration in pulmonary functions in animal and human subjects.

In considering problems relating to the development of more serious chronic diseases, cancer induction appears to be the most important indicated. Carcinogenic materials have been identified in the atmosphere of all large cities of the world. The incomplete combustion of organic materials is the major source of a variety of carcinogenic polynuclear aromatic hydrocarbons, of which benzo(a)pyrene is a prime example. The experimental induction of carcinomas in rats has been demonstrated by combined inhalation of benzo(a)-pyrene and sulfur dioxide. These facts, along with the knowledge that oxides of sulfur and their corresponding acids and salts are major common pollutants, have formed the basis for studies with these compounds in combination with known carcinogens.

A model has been developed in which hamsters were exposed to a carcinogen by intratracheal-intubation and to sulfur dioxide by inhalation. The finding of cancer in these studies reinforces the direct inhalation studies reported from rats by other investigators.

This study will utilize the intubation-inhalation technique for the exposure of male Syrian Golden hamsters to a carcinogen followed by inhalation exposures to the various co-factors. These co-factors include sulfuric acid mist and other sulfur oxides, alone and in combination with aerosols. Because of the complexity of the developmental work required for these studies, the program is divided into several phases.

Phase I involves range-finding studies to determine tolerable exposure levels of sulfuric acid mist. Phase II includes combined intubation-inhalation studies with benzo(a)pyrene and sulfuric acid mist. Phase III involves the addition of other co-factors alone or in conjunction with sulfuric acid mist. Phase IV involves the inhalation exposure of animals to the by-products of sulfur dioxide passed through a vanadium pentoxide catalytic converter.

In the development of the inhalation studies, the primary considerations were the generation of atmospheres which could be controlled with respect to mass, concentration, and particle size distribution. In the method of generation used, concentrated sulfuric acid was spray-atomized against the walls of a 500 ml glass baffle chamber, using a stainless steel nebulizer developed in this laboratory. The aerosol was then introduced into the air intake of the 1.3 cubic meter animal exposure chamber. Particle size and concentration could be controlled by variations in baffle characteristics, spray pressure and acid concentration.

Chamber concentrations were routinely sampled, using a midget impinger, at half-hour intervals during exposure. The analytical technique involved titrating the sample with a standardized sodium hydroxide solution, using methyl red as the indicator. The analytical precision of this method, as determined by 9 replicate samples, is better than ± 0.001 mg/ml which corresponds to a precision of ± 0.5 mg/m^3 in the chamber atmosphere. The sampler efficiency, as determined by 10 repetitive tandem samples, is 94.5% with a standard deviation of 0.3%. Repeated tests showed that interference from carbon dioxide is negligible.

Alternate analytical techniques were used occasionally as independent verification of chamber concentration. These techniques are ASTM D:516, non-referee methods A and B, for the determination of sulfate.

Extensive studies were performed to characterize the particle size distribution of the sulfuric acid mist. The importance of such a determination is well known, since this defines the site of deposition of the aerosol and its physiochemical behavior. Several measurements of the particle size distribution of the sulfuric acid mist were made using the modified Casella Cascade impactor and the Andersen Model 10-000 impactor. A significant disagreement was observed when the two distributions were compared. It was, therefore, necessary to verify the calibration of these two instruments, using the ultramicroscope and the Royco Model 202 particle counter.

The hydroscopic nature of sulfuric acid mist and its corrosive properties indicated a need for a more stable aerosol for comparative calibration. An alternate aerosol of 10% glycerine in water was selected, incorporating flourescein as an analytical tag. The particle size distributions obtained for this aerosol by the Casella and the Andersen impactors were similar to those obtained for the sulfuric acid mist. Also, the same variations between the two methods were observed.

Optical determinations using the ultramicroscope were made on 497 particles. The parameters of the distribution obtained with the glycerine-water aerosol were a mean aerodynamic diameter of 1.6 μm with a geometric standard deviation of 1.5. The distribution parameters obtained by the Casella impactor were a mean diameter of 1.8 μm with a geometric standard deviation of 1.8 and those for the Andersen impactor were a mean diameter of 0.9 with a geometric standard deviation of 1.8. It appears that a correlation exists between the mean diameters obtained by the ultra-microscope and Casella type impactor.

Particle size distributions were obtained simultaneously with the Royco particle counter and samples were collected by the Casella and the Andersen impactors. The results obtained suggested that the count median diameter obtained by the Royco corresponds very closely with the median obtained by the Andersen impactor.

The range finding study consisted of a single 6-hour exposure of a group of 10 Syrian Golden hamsters to concentrations of 300 and 500 mg/m^3 of sulfuric acid mist. Based on the observations of the single exposure studies, a 30-day inhalation study was conducted at a concentration of 100 mg/m^3. Forty male hamsters were exposed for 6 hours per day, 5 days per week. Twenty colony controls were selected to parallel this study.

Segment 1 of the Phase II combined intubation-inhalation studies involved a single intubation of relatively high levels of carcinogen (10 mg or 40 mg B[a]P) followed by a life-time exposure to sulfuric acid mist. The experimental outline for Segment 1 is given in Table 1. The suspension of B[a]P alone and in combination with other materials were prepared by ball mill grinding. Particle size determinations of the suspensions were performed by optical microscopy. The geometric count median diameter was found to be 2.1 μm with a standard deviation of 2.3.

Segment 2 (Phase II) involved the multiple intubation of 1 and 4 mg B[a]P on a schedule of 1 intubation per week for 15 consecutive weeks. Following the first intubation, the animals were exposed for life to 100 mg/m^3 sulfuric acid. Control groups were included. The experimental outline for Segment 2 is given in Table 2.

In order to generate a multicompartment atmosphere, a catalytic reactor was designed and developed to convert SO_2 to other sulfur oxides. The reactor consists of a temperature-regulated oven capable of maintaining constant compartmental temperature, a tubular catalyst bed, and a 13.5 liter reservoir from which samples are periodically drawn for analysis. Sulfur dioxide concentrations entering the reactor are regulated by metering known volumes of compressed air and SO_2. In order to determine relative percent conversion of SO_2, bubbler samples for SO_2 and total SO_x were collected from the reservoir. To optimize the SO_2 conversion, the effects of the catalyst composition, gas temperatures, and sulfur dioxide concentrations were examined. After optimizing the SO_2 conversion conditions, characterization of the oxide product will be accomplished using spectrophotometry, gas chromatography, and mass spectrometry. Tests will be conducted at increased humidities to determine the extent and particle size of sulfuric acid mist resulting from SO_2 conversion. Subsequent to these analyses, an aerosolized material will be generated into the reservoir along with the sulfur oxides and the determination of resultant products will be made. The aerosolized material will consist of metals, metal oxides, carbon and combinations of these materials as available. Where feasible, animal studies will be designed and performed to characterize the toxicity of suspect product mixtures.

Research Accomplished

Range finding experiments were completed, and on the basis of the results obtained, a concentration of 100 mg/m^3 of sulfuric acid mist was selected for the combined intubation-inhalation studies. The initial responses of the animals to the single exposure experiment were nasal and eye irritation, slight dyspnea, and a body weight loss of 3 to 4 grams for approximately one week. Subsequent weight gain began to parallel that of the controls, but the initial loss was not recovered. No deaths occurred in animals exposed to either concentration. Two animals per exposure group were sacrificed at the end of the 15th experimental day. One of the sacrificed animals from each of the exposure groups was found to have partial atelectasis and focal emphysema. The remaining animals from both groups were sacrificed on the 30th day post-exposure. All showed slight thickening of the alveolar septa, irrespective of exposure concentration. However, there was no abnormality observed in the bronchial epithelium. Based on the observations of the single exposure studies, a concentration of 100 mg/m^3 of sulfuric acid mist was selected for the 30-day inhalation study. In this study, 40 male hamsters were exposed for 6 hours per day, 5 days per week. Twenty colony controls were selected to parallel this study. During the initial 1 to 2 weeks of exposures, the animals exhibited slight respiratory irritation. Thereafter, the animals appeared to have adapted to the experimental atmosphere. The weight gain of the experimental animals was depressed following the first week of exposure. Thereafter, the weight gain of the exposed animals was comparable to that of controls. No deaths occurred during the 30-day sulfuric acid mist exposures. These animals were kept for further observation. Interim sacrifice of 5 hamsters was performed on the 57th experimental day. Histopathological examination of the lungs revealed no exposure related abnormalities. Major lung histology and mucosal changes appeared to be congestion, hemorrhage, and edema. No significant differences in findings in the bronchial or broncho-alveolar region were observed. Of interest, however, were the findings of 2 largyngeal hyperplasias and 2 laryngeal squamous metaplasias in animals exposed to sulfuric acid mist.

The chronic studies (Phase II) involving the combined intubation-inhalation exposures with benzo(a)pyrene (B[a]P) and sulfuric acid mist are nearing completion. The experimental outlines for Phase II are shown in Tables 1 and 2. The first segment of this phase involved single intubations of B[a]P followed by lifetime exposures to sulfuric acid mist. The cumulative data to date indicates that all of the animals had died by 116 weeks in Segment 1. Weight change in animals receiving intubations alone was markedly lower than that of the animals receiving sulfuric acid mist exposure. Weight change of the air controls was similar to sulfuric acid mist exposure groups, while the weight change of the untreated controls was similar to that of the groups receiving intubations alone.

Major lung histology for this segment is shown in Table 3 and includes early findings of hemorrhage and edema. The major mucosal changes are given in Tables 4 and 5.

Three benign and two malignant tumors were found in the respiratory tract of animals receiving 40 mg B[a]P alone, compared to one benign tumor in the group receiving both 40 mg B[a]P and sulfuric acid. In the group receiving 10 mg B[a]P once with sulfuric acid, two out of 45 animals developed malignant tumors and one developed a benign tumor in the lung, whereas the animals receiving 10 mg B[a]P alone developed only one benign tumor in the lung. The highest tumor incidence was seen in the positive group receiving 4 mg B[a]P with 4 mg ferric oxide where 11 of 30 animals (37%) developed tumors in the respiratory tract. The tumor incidences for all groups are given in Table 6. The detailed morphological types are described in Table 7. Non-respiratory tract tumors have also been observed and these findings are given in Table 8.

All animals receiving multiple intubations (Segment 2) with or without sulfuric acid mist exposures were dead by the 100th week. In the remaining groups, all animals were dead by the 120th week with the exception of one animal from the group receiving sulfuric acid alone. This animal was still alive and being exposed at the 128th week, although loss of weight was evident from the 120th week onward. The histological findings for this segment are shown in Table 9. Major mucosal findings are summarized in Tables 10 and 11. A high percentage of tumors in the respiratory tract were found in animals receiving the multiple dose intubations of B[a]P (4mg B[a]P x 15) with or without sulfuric acid mist. A slight increase in the tumor incidences was found in animals receiving B[a]P alone. Eighty percent of the animals in the group receiving B[a]P alone showed tumors in the respiratory tract compared to 72% in the animals receiving both B[a]P and sulfuric acid mist. In contrast, in the group receiving the lower dose (1 mg B[a]P x 15), a much higher incidence of respiratory tract tumors was seen when sulfuric acid mist was given (24%) than when the B[a]P was given alone (12%).

The segmental distribution in the respiratory tract and morphological classifications of the individual tumors are given in Tables 12 and 13. The non-respiratory tract tumors found in these groups are listed in Table 14.

The conversion efficiency of a vanadia molybdate catalyst at higher concentrations of SO_2 was evaluated. At ambient temperature or at 400°C, the molybdate catalyst does not appear to increase the conversion substantially. Upon an average input of 64.2 ppm SO_2, at ambient temperature, it varies from 2.9 to 11.4%. At 400°C, it varies from 3.0 to 8.8% When moisture was introduced into the system, it was 7.9% at ambient and 10.0% at 400°C. Neither absorption nor desorption were observed.

In summary, the vanadia molybdate catalyst is not as efficient as the vanadium pentoxide catalyst in converting SO_2 to higher state sulfur oxides. Elevated temperature, moisture and high concentration of SO_2 do not appear to increase the conversion efficiency.

Since the vanadium pentoxide catalyst appears to be most effective in converting SO_2 to other sulfur oxides among three catalysts evaluated, this catalyst was used to oxidize SO_2 to other sulfur oxides, at 400°C. with moisture. The products of this process were then analyzed by using several methods, such as thermometric methods, gas chromatography, mass spectroscopy, spectrophotometry and conventional wet chemistry. Iron oxide particulates also introduced into the system during this period, since iron oxide particulates are abundant in the atmosphere and are capable of oxidizing SO_2.

The iron oxide aerosol was generated using a DeVilbiss nebulizer and introduced into the 13.5 liter reservoir, mixed with the gases which had been passed over the catalyst. The particulates were then collected on two successive millipore membrane filters and subsequently analyzed for the sulfur species on the filter by thermometric methods. The gaseous products were analyzed by the iodine titration and thermometric methods and the characterization will be accomplished using gas chromatography, mass spectroscopy and spectrophotometry.

The iron oxide particulates produced by the DeVilbiss nebulizer were collected on the microscopic grid by a point to plane electrostatic precipitator developed by this laboratory. The particles were counted and sized by scanning electron microscopy. The iron oxide appears to be mostly spheres. It has a mass median diameter of 0.34 μm, a surface median diameter of 0.2 μmo, and a count median diameter of 0.02 μm with og of 1.29.

Later in this study, the particulates will be mixed with the gases coming out from the catalyst, and the efficiency and products will be analyzed.

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Related Research

A number of toxocological studies using various sulfur compounds of complexes have been conducted which relate to the problem of acute and chronic toxicity.

TABLE 1 EXPERIMENTAL OUTLINE FOR COMBINED INTUBATION-INHALATION STUDIES*
PHASE 2 - SEGMENT 1

Number of Animals	Intubation	Inhalation
60	40 mg/0.2 cc BP x 1	+ 100 mg/m^3 H_2SO_4 x life
60	40 mg/0.2 cc BP x 1	---
60	10 mg/0.2 cc BP x 1	+ 100 mg/m^3 H_2SO_4 x life
60	10 mg/0.2 cc BP x 1	---
60	0.1 mg/0.2 cc Gel x 1	+ 100 mg/m^3 H_2SO_4 x life
60	0.1 mg/0.2 cc Gel x 1	---
60	---	100 mg/m^3 H_2SO_4 x life
60	Air Control	---
60	Colony Control	---
30	4 mg/0.2 cc BP + Fe_2O_3 x 15	---
30	1 mg/0.2 cc BP + Fe_2O_3 x 15	---

* Repeat Series

TABLE 2 EXPERIMENTAL OUTLINE FOR COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2

Number of Animals	Intubation	Inhalation
60	4 mg/0.2 cc BP x 15	+ 100 mg/m^3 H_2SO_4 x life
60	4 mg/0.2 cc BP x 15	---
60	1 mg/0.2 cc BP x 15	+ 100 mg/m^3 H_2SO_4 x life
60	1 mg/0.2 cc BP x 15	---
60	0.1 mg/0.2 cc Gel x 15	+ 100 mg/m^3 H_2SO_4 x life
60	0.1 mg/0.2 cc Gel x 15	---
60	---	100 mg/m^3 H_2SO_4 x life
60	Air Control	---
60	Colony Control	---
30	4 mg/0.2 cc BP + Fe_2O_3 x 15	---
30	1 mg/0.2 cc BP + Fe_2O_3 x 15	---

TABLE 3 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 1 - SINGLE INTUBATION SERIES
MAJOR LUNG HISTOLOGY*

Inhalation[a]	Intubation	Hemorrhage Congestion Edema	Pneumonia	Infiltration
H_2SO_4	40 mg BP x 1	50/56	23/56	0/56
---	40 mg BP x 1	54/60	31/60	5/60
H_2SO_4	10 mg BP x 1	41/45	20/45	0/45
---	10 mg BP x 1	58/59	26/59	1/59
H_2SO_4	0.1 mg Gel x 1	58/59	26/59	1/59
---	0.1 mg Gel x 1	58/60	43/60	0/60
---	4 mg BP + 4 mg Fe_2O_3**	25/30	16/30	2/30
---	1 mg BP + 1 mg Fe_2O_3*	29/29	13/29	0/29
H_2SO_4	---	58/60	27/60	0/60
---	Air Control	58/59	38/59	1/69
---	Colony Control	50/57	26/57	4/57

*Denominator shows ovservations to date

[a] H_2SO_4 100 mg/m^3 x life

*15 intubations

TABLE 4 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 1 - SINGLE INTUBATION SERIES
MAJOR UPPER RESPIRATORY TRACT MUCOSAL CHANGES**

Type of Exposure		Laryngeal		Trachael	
Inhalation[a]	Intubation	Hyperplasic	Squamous Metaplasia	Hyperplasis	Squamous Metaplasia
H_2SO_4	40 mg BP x 1	9/56	1/56	13/56	0/56
---	40 mg BP x 1	9/58	0/58	6/58	0/58
H_2SO_4	10 mg BP x 1	7/42	0/42	1/45	0/45
---	10 mg BP x 1	7/57	1/57	3/59	0/59
H_2SO_4	0.1 mg Gel x 1	34/57	2/57	17/59	0/59
---	0.1 mg Gel x 1	4/58	0/58	4/60	0/60
---	4 mg BP + 4 mg Fe_2O_3	4/29	0/29	4/30	0/30
---	1 mg BP + 1 mg Fe_2O_3	2/28	0/28	0/29	0/29
H_2SO_4	---	16/58	0/58	14/60	0/60
---	Air Control	12/59	0/59	7/59	0/59
---	Colony Control	11/57	0/57	10/57	0/57

*Denominator shows observations to date

[a] H_2SO_4 100 mg/m^3 x life

**15 Intubations

TABLE 5 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 1 - SINGLE INTUBATION SERIES
MAJOR LOWER RESPIRATORY TRACT MUCOSAL CHANGES*

Type of Exposure		Bronchial		Broncho-Alveolar	
Inhalation[a]	Intubation	Hyperplasia	Squamous Metaplasia	Hyperplasia	Squamous Metaplasia
H_2SO_4	40 mg BP x 1	41/56	0/56	0/56	0/56
---	40 mg BP x 1	44/60	0/60	7/60	0/60
H_2SO_4	10 mg BP x 1	33/45	0/45	1/45	0/45
---	10 mg BP x 1	54/59	0/59	5/59	0/59
H_2SO_4	0.1 mg Gel x 1	50/59	0/59	2/59	0/59
---	0.1 mg Gel x 1	42/60	0/60	1/60	0/60
---	4 mg BP + 4 mg Fe_2O_3**	22/30	3/30	9/30	2/30
---	1 mg BP + 1 mg Fe_2O_3**	17/29	1/29	5/29	0/29
H_2SO_4	---	41/60	1/60	4/60	0/60
---	Air Control	47/59	0/59	1/59	0/59
---	Colony Control	33/57	0/57	2/57	0/57

*Denominator shows observations to date

[a] H_2SO_4 100 mg/m^3 x life

**15 intubations

TABLE 6 INTUBATION-INHALATION EXPOSURES WITH HAMSTERS
PHASE 2 - SEGMENT 1

		Respiratory Tract Tumors of:			
Type of Exposure	TBA/Number Observed	Larynx	Trachea	Lungs	Other
40 mg BP x 1 + H_2SO_4 x Life	1/56 (2%)	0	1	0	2
40 mg BP x 1	5/60 (8%)	1	2	2	7
10 mg BP x 1 + H_2SO_4 x Life	2/45 (4%)	0	0	3	3
10 mg BP x 1	1/59 (2%)	0	0	1	10
0.1 mg Gel x 1 + H_2SO_4 x Life	0/42 (0%)	0	0	0	3
0.1 mg Gel x 1	0/60 (0%)	0	0	0	2
4 mg BP + 4 mg Fe_2O_3 x 15	11/30 (37%)	0	2	10	10
1 mg BP + 1 mg Fe_2O_3 x 15	2/30 (7%)	0	0	2	5
100 mg/m^3 H_2SO_4 x Life	0/60 (0%)	0	0	0	6
Air Control x Life	0/59 (0%)	0	0	0	1
Untreated Control	0/57 (0%)	0	0	0	7

TABLE 7 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 1 - SINGLE INTUBATION SERIES

Type of Exposure	No. Tumor-Bearing Animals No. Animals Observed	Type of Tumor	Day First Tumor Observed
40 mg BP + H_2SO_4 x Life	1/56	1 Polyp-Trachea	353
40 mg BP	5/60	2 Papilloma-Trachea	477
		1 Papilloma-Larynx	577
		1 Adenocarcinoma-Lung	705
		1 Undiff.-Car.-Lung	722
10 mg BP + H_2SO_4 x Life	2/45	1 Adenoma-Lung	286
		1 Sq. Cell Car.-Lung	286
		1 Adenocarcinoma-Lung	506
10 mg/ BP x 1	1/59	1 Adenoma-Lung	538
4 mg BP + 4 mg Fe_2O_3 x 15	11/30	1 Papilloma-Trachea	366
		1 Polyp-Trachea	504
		3 Sq. Cell Car.-Lung	338
		3 Adenocarcinomas-Lung	389
		3 Mixed Carcinomas-Lung	367
		1 Adenoma-Lung	481
1 mg BP + mg Fe_2O_3 x 15	2/30	1 Sq. Cell Car.-Lung	423
		1 Adenocarcinoma-Lung	324

TABLE 8 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 1 - SINGLE INTUBATION SERIES

	Non-Respiratory Tract Tumors	
Type of Exposure	Type of Tumor	Day First Tumor Observed
40 mg BP x 1 + H_2SO_4 x Life	1 Papilloma-Fore-Stomach	419
	1 Malignant Lymphoma	527
40 mg BP x 1	1 Malignant Lymphoma	395
	6 Papillomas-Fore-Stomach	480
10 mg BP x 1 + H_2SO_4 x Life	3 Papillomas-Fore-Stomach	384
10 mg BP x 1	1 Malignant Lymphoma	329
	9 Papillomas-Fore Stomach	382
4 mg BP + 4 mg Fe_2O_3 x 15	8 Papillomas-Fore-Stomach	225
	1 Malignant Lymphoma	481
	1 Adrenal-Cortical Adenoma	389
1 mg BP + 1 mg Fe_2O_3 x 15	1 Malignant Lymphoma	435
	2 Papillomas-Fore-Stomach	629
	1 Adrenal-Cortical Adenoma	514
	1 Fibromatosis-Sub-cutaneous	629
0.1 mg Gel x 1 + H_2SO_4 x Life	2 Papillomas-Fore-Stomach	634
	1 Adrenal-Cortical Adenoma	598
0.1 mg Gel x 1	2 Papilloma-Fore-Stomach	634
100 mg/m^3 H_2SO_4 x Life	1 Malignant Lymphoma	114
	2 Papilloma-Fore-Stomach	503
	1 Adrenal-Cortical Adenoma	568
	1 Fibroma	657
Air Control	1 Papilloma-Fore-Stomach	668
Untreated Control	5 Papillomas-Fore-Stomach	690
	1 Adrenal Cortical Adenoma	675
	1 Malignant Lymphoma	702

TABLE 9 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2 - MULTIPLE INTUBATION SERIES
MAJOR LUNG HISTOLOGY*

Inhalation[a]	Intubation[b]	Hemorrhage Congestion Edema	Pneumonitis	Lymphocytic Infiltration
H_2SO_4	4 mg BP	51/60	34/60	0/60
---	4 mg BP	41/50	31/60	0/60
H_2SO_4	1 mg BP	51/58	27/58	1/58
---	1 mg BP	56/59	40/59	3/59
H_2SO_4	0.1 mg Gel	56/59	32/59	2/59
---	0.1 mg Gel	49/55	33/55	4/55
---	4 mg BP + 4 mg Fe_2O_3	26/30	17/30	2/30
H_2SO_4	---	52/60	26/60	0/60
---	Air Control	58/60	41/60	0/60
---	Colony Control	51/58	24/58	6/58

*Denominator shows observations to date

[a] H_2SO_4 100 mg/m^3 x life

[b] 15 intubations

TABLE 10 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2 - MULTIPLE INTUBATION SERIES
MAJOR UPPER RESPIRATORY TRACT MUCOSAL CHANGES*

Type of Exposure		Laryngeal		Tracheal	
Inhalation[a]	Intubation[b]	Hyperplasia	Squamous Metaplasia	Hyperplasia	Squamous Metaplasia
H_2SO_4	4 mg BP	7/59	5/59	13/50	0/60
---	4 mg BP	15/60	0/60	15/60	1/60
H_2SO_4	1 mg BP	7/55	1/55	7/58	0/58
---	1 mg BP	9/55	0/55	7/58	0/58
H_2SO_4	0.1 mg Gel	12/59	1/59	7/59	0/59
---	0.1 mg Gel	5/54	0/54	4/54	0/54
---	4 mg BP + 4 mg Fe_2O_3	8/29	1/29	5/29	0/29
---	1 mg BP + 1 mg Fe_2O_3	7/29	0/29	5/29	0/29
H_2SO_4	---	11/57	0/57	7/60	0/60
---	Air Control	12/58	1/58	8/59	0/59
---	Colony Control	9/58	0/58	7/58	0/58

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* Denominator shows observations to date.

[a] H_2SO_4 100 mg/m^3 x life

[b] 15 intubations

TABLE 11 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2 - MULTIPLE INTUBATION SERIES
MAJOR LOWER RESPIRATORY TRACT MUCOSAL CHANGES*

Type of Exposure			Bronchial	
Inhalation[a]	Intubation[b]	Hyperplasia	Squamous Metaplasia	Metaplasia
H_2SO_4	4 mg BP	38/60	6/60	21/60
---	4 mg BP	37/60	10/60	28/60
H_2SO_4	1 mg BP	46/58	0/58	30/58
---	1 mg BP	51/59	0/59	27/59
H_2SO_4	0.1 mg Gel	38/59	1/59	2/59
---	0.1 mg Gel	38/55	0/55	1/55
---	4 mg BP + 4 mg Fe_2O_3	24/30	1/30	12/30
---	1 mg BP + 1 mg Fe_2O_3	26/29	0/29	4/29
H_2SO_4	---	44/60	0/60	1/60
---	Air Control	39/60	0/60	2/60
---	Colony Control	37/58	0/58	2/58

---

* Denominator shows observations to date.

[a] H_2SO_4 100 mg/m^3 x life

[b] 15 intubations

TABLE 12 INTUBATION-INHALATION EXPOSURES WITH HAMSTERS
PHASE 2 - SEGMENT 2

		Respiratory Tract Tumors of:		
Type of Exposure	TBA/Number Observed	Larynx	Trachea	Lungs
4 mg BP x 15 + H_2SO_4 x Life	43/60 (72%)	0	5	47
4 mg BP x 15	48/60 (80%)	0	7	58
1 mg BP x 15 + H_2SO_4 x Life	14/58 (24%)	0	3	13
1 mg BP x 15	7/59 (12%)	0	1	6
0.1 mg Gel x 15 + H_2SO_4 x Life	0/59 (0%)	0	0	0
0.1 mg Gel x 15	1/55 (2%)	0	1	0
4 mg BP + 4 mg Fe_2O_3 x 15	8/30 (27%)	1	0	7
1 mg BP + 1 mg Fe_2O_3 x 15	2/29 (7%)	0	1	1
100 mg/m^3 H_2SO_4 x Life	0/60 (0%)	0	0	0
Air Control	0/60 (0%)	0	0	0
Untreated Control	0/58 (0%)	0	0	0

TABLE 13 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2 - MULTIPLE INTUBATION SERIES

		Respiratory Tracr Tumors	
	Number of/ Tumor-/ Number of Bearing/Animals Animals/ Observed	Type of Tumor	Day First Tumor Observed
4 mg BP x 15 + H_2SO_4	43/60	28 Adenocarcinomas-Lung	270
		9 Sq. Cell Car.-Lung	292
		6 Mixed Carcinomas	358
		3 Adenomas-Lung	394
		3 Papillomas-Trachea	371
		2 Polyp-Trachea	387
		1 Frirosarcoma-Lung	389
4 mg BP x 15	48/60	3 Adenomas-Lung	341
		29 Adenocarcinomas-Lung	295
		14 Sq. Cell Car.-Lung	244
		11 Mixed Carcinomas-Lung	325
		1 Sq. Cell Car.-Trachea	407
		3 Papillomas-Trachea	387
		1 Undiff. Car.-Trachea	347
		1 Papilloma-Bronchus	474
		1 Anaplastic Car.-Trachea	534
		1 Polyp-Trachea	600
1 mg BP x 15 + H_2SO_4 x Life	14/58	6 Adenocarcinomas-Lung	310
		6 Adenomas-Lung	345
		1 Adenocarcinoma-Trachea	481
		2 Papilloma-Trachea	544
		1 Anaplastic Car.-Lung	697
1 mg BP x 15	7/59	4 Adenocarcinoma-Lung	399
		1 Mixed Carcinoma-Lung	336
		1 Adenoma-Lung	448
		1 Polyp-Trachea	602
4 mg BP + 4 mg Fe_2O_3 x 15	8/30	3 Adenocarcinoma-Lung	500
		5 Sq. Cell Car.-Lung	280
		1 Papilloma-Larynx	392
		1 Mixed Car.-Lung	621
1 mg BP + 1 mg Fe_2O_3 x 15	2/29	1 Adenoma-Lung	318
		1 Papilloma-Trachea	653
0.1 mg Gel x 15	1/55	1 Papilloma-Trachea	674

TABLE 14 COMBINED INTUBATION-INHALATION STUDIES
PHASE 2 - SEGMENT 2 - MULTIPLE INTUBATION SERIES

	Non-Respiratory Tract Tumors	
Type of Exposure	Type of Tumor	Day First Tumor Observed
4 mg BP x 15 + H_2SO_4 x Life	19 Pipillomas-Fore-Stomach	270
	4 Malignant Lymphomas	270
	1 Fibrosarcoma-S.C.	275
	1 Adenocarcinoma-Kidney	515
	1 Adenocarcinoma-Adrenal	387
	1 Hemangioma-Spleen	421
4 mg BP x 15	22 Papillomas-Fore-Stomach	273
	1 Fibrosarcoma	266
	1 Sq. Cell Car.-Fore-Stomach	348
	2 Hemangioma-Spleen	335
	1 Fibroma-S.C.	428
	1 Malignant Lymphoma	457
1 mg BP x 15 + H_2SO_4 x Life	7 Papillomas-Fore-Stomach	424
	1 Malignant Lymphoma	440
1 mg BP x 15	9 Papillomas-Fore-Stomach	283
	2 Malignant Lymphoma	263
4 mg BP + 4 mg Fe_2O_3 x 15	13 Papillomas-Fore-Stomach	310
	2 Malignant Lymphomas	387
1 mg BP + 1 mg Fe_2O_3 x 15	5 Papillomas-Fore-Stomach	600
	1 Neuroblastoma-Adrenal	531
0.1 mg Gel x 15 + H_2SO_4 x Life	1 Papilloma-Fore-Stomach	610
	1 Malignant Lymphoma	572
0.1 mg Gel x 15	2 Adenomas-Adrenal	537
	2 Papillomas-Fore-Stomach	481
100 mg/m^3 H_2SO_4 x Life	2 Papillomas-Fore-Stomach	473
	1 Hamangioma-Spleen	556
Air Control	3 Malignant Lymphomas	440
	2 Papilloma-Fore-Stomach	658
	1 Adenoma-Adrenal	461
Colony Control	1 Malignant Lymphoma	348
	1 Hamangioma-Spleen	420
	1 Papilloma-Fore-Stomach	626

E. TASK TITLE:

Compare Effects of Respirable Particles, Gases and Mists Using Small Airway Resistance in Donkeys as the Model for Pulmonary Irritation

HERL/RTP TASK NO: 8199

CONTRACTOR: New York University

CONTRACT NO.: 68-02-1732

Summary

The investigations of the effects of $(NH_4)_2SO_4$ and H_2SO_4 aerosols on pulmonary function, regional deposition, and bronchial clearance will be pursued in order to clearly establish the nature of the effects produced and their dose-response relationships. This will provide a sound basis for subsequent tests with the same aerosols on human volunteers.

Airway resistance, dynamic compliance, and regional particle deposition were selected as indicators of physiological response. They will indicate transient effects attributable to the inhalation of airborne irritants at levels which produce no pathological effects. Effects on these physiological functions in donkeys should be essentially similar to those produced by the same irritants in humans. A major purpose is, therefore, to clarify the dose-response relationship for pulmonary irritation resulting from transient elevations in the ambient pollution aerosol concentration.

The intrabronchial deposition patterns of $(NH_4)_2SO_4$ and H_2SO_4 aerosols will also be measured in hollow bronchial casts of donkeys and human airways. The effect of water vapor concentration on the rate of aerosol growth and subsequent deposition in hollow casts will be tested in order to evaluate mathematical predictions of droplet growth from physiochemical factors.

Scope and Objectives

Changes in pulmonary function, mucociliary clearance, and the regional deposition of an inert test aerosol will be studied to characterize the dose-effects relationships produced in donkeys by inhaled sulfuric acid and ammonium sulfate aerosols. The donkey will be exposed without sedation or rigid restraint and represents an analogue for man. The human dose-response to a one hour exposure at low concentrations (<1 mg/m^3) of sulfuric acid mists 0.5 μm in diameter will be evaluated. Measurements will be made of pulmonary function, mucociliary transport and aerosol deposition.

Research Accomplished

Animal Studies--Two animals have undergone six month exposure to 100 μg/m^3 H_2SO_4 acid mist (0.3 μm) for one hour a day for five days a week. Two additional donkeys have been evaluated for normal (base line) measurements and are presently undergoing an identical six month exposure regimen. Bronchial clearance rates were slower initially and were faster thereafter. However, there is considerable variation and no definite pattern has emerged.

Human Studies--Human testing has commenced with each subject being exposed to 0, 100, 300, or 1000 $\mu g/m^3$ of H_2SO_4 in a random fashion in a blind test. Studies of the second human volunteer are presently underway.

Related Research

This project is complimentary to the Research activities in the Clinical Studies Division in Chapel Hill, North Carolina.

F. TASK TITLE:

Studies on the Relationship Between Carcinogen Metabolism in the Alveolar Macrophage and the Induction of Lung Cancer

HERL/RTP TASK NO:	8157
CONTRACTOR:	Northrop Services, Inc.
CONTRACT NO.:	68-02-2566

Summary

Rabbit alveolar macrophages were examined for their ability to metabolize or activate procarcinogens and promutagens to their active forms. It has been demonstrated that rabbit alveolar macrophage can activate the mutagen 2-anthramine using the Salmonella typhimurium bacterial mutagenesis assay of Ames. This activity is within an order of magnitude of that found in rat liver, a startling finding. This metabolic activity is mediated be NADPH and oxygen and is partially located in the endoplasmic reticulum (microsomal fraction) of the macrophage.

Primary rat hepatocytes have been used to establish a cell mediated bacterial mutagenesis bioassay using strains of Salmonella as the indicator organism. This bioassay is sensitive to a number of hepatocarcinogens, indicating that it may possess organotropic specificity. Benzo(a)pyrene has been found to be negative in this system and the explanation for this phenomena is being sought through metabolism studies using high pressure liquid chromatography. Once the techniques and protocols for this new assay have been established they will be applied to cocultivation of rabbit alveolar macrophage and Salmonella.

Scope and Objective

The objective of this task is to study the relationship between carcinogen metabolism in the alveolar macrophage and the induction of lung cancer. Using *in vitro* methods the course of metabolic activation/detoxification of procarcinogens will be investigated in alveolar macrophages obtained from rats and hamsters. Biochemical methods will be used in monitoring induction of metabolic activation/detoxification systems. Microbial mutagenesis will be used to monitor the extent of metabolic activation/detoxification by macrophages. Once the conditions for maximal activation/detoxification of carcinogens have been established using in vitro methods, whole animal studies will be initiated employing intratracheal instillation techniques to administer particulates and appropriate procarcinogens. The metabolic activation of procarcinogens will be monitored as described above using macrophages obtained by saline lavage of carcinogen-exposed animals. When optimal conditions have been established, experiments will be performed to demonstrate the relationship between elicitation of macrophage influx, metabolic activation/detoxification of carcinogens and the induction of lung tumors in animals. This is a long-range project that will be approached sequentially with continuation from year to year being dependent upon demonstrated success of initial studies.

Background and Approach

Pioneering work by Safiotti and others has demonstrated the importance of the particulate component in experimental pulmonary carcinogenesis in rodent models. Ambient air particulate is known to contain procarcinogenic compounds such as benzo(a)pyrene. The alveolar macrophage constitutes the first line of defense against inhaled particulate, having been shown many years ago to avidly phagocytize foreign materials including air particulates which find their way to the deep lung. The half-life of particles which penetrate to this level of the respiratory tract is months to years. Therefore, the alveolar macrophage is in a unique position to sequester and potentially to metabolically activate procarcinogenic compounds associated with airborne particulate material. Using in vitro methods as previously described, it is possible to determine whether the macrophage is competent enzymatically to carry out metabolic activation and to determine whether activated compounds are released from the intact macrophage to cause damage to the DNA of adjacent cells. Thence, it should be possible to determine, under optimal conditions, where cancer incidence is greater in experimental animals.

Research Accomplished

Initial studies with procarcinogens utilizing in vitro methodology are investigating the metabolic activation and detoxification capabilities of the alveolar macrophage. Experiments performed thus far demonstrate that a microsomal fraction from alveolar macrophages is capable of metabolizing the procarcinogen 2-aminoanthracene (2-anthramine) to a mutagenically active species. Macrophages stimulated with Bacillus-Calmette-Guerin were found to have less metabolic activation capability when tested with anthramine in the *Salmonella typhimurium* bacterial mutagenesis bioassay. Biochemical assays for aryl hydrocarbon hydroxylase, cytochrome P-450 and other microsomal enzymes show the activity to be several orders of magnitude below that found in liver. However, the mutagenic activity of 2-aminoanthracene using macrophage S-9 preparation was within an order of magnitude of that obtained from liver.

The question was raised whether the activation of anthramine by rabbit alveolar macrophage (RAM S-9) is mediated by NADPH and O_2 requiring mixed-function oxidases? This question has not been completely resolved. In the absence of NADPH the reversion rate returned to the spontaneous (background) levels. A 100,000 xg supernatant prepared from the S-9 had about the same activity as the microsomes isolated from the same spin on a per mg protein basis. The total activity of the 100,000 xg supernatant and the microsomes was about equal to the S-9 from which it was prepared. Hence, the activation of anthramine requires NADPH, but the enzymes effecting this process are not solely located in the microsomes. It was determined that RAM S-9 also activates 2-aminofluorene but attempts to activate benzo-a-pyrene were unsuccessful.

Since macrophages had the ability to activate promutagens to their active mutagenic forms, the development of a cell-mediated bioassay system using macrophage as the metabolizer cell and _Salmonella typhimurium_ as the indicator cell line was undertaken. Due to the difficulty in obtaining macrophages at that time, primary rat hepatocytes were substituted in their place as the technical problems associated with cell mediated mutagenesis bioassays would be somewhat the same whether macrophage or hepatocytes were used. Primary rat hepatocytes were then used to establish a system for utilizing microbial mutagenesis in screening for carcinogenic metabolites of whole cells. _S. typhimurium_ strain TA1538 is placed in a dialysis bag and incubated with the hepatocytes and mutagen. The bacteria are then removed, plated on minimal media, and scored 2 days later for revertant colonies. Incubation of increasing numbers of hepatocytes with anthramine and Ta1538 resulted in increasing numbers of revertant colonies. Incubation times were varied with 1 hour determined to be best. Dose curves were constructed using fixed cell concentrations and varying anthramine concentrations. Number of revertants was a function of anthramine dose. Studies are underway to determine the optimum number and volume of cells needed for the cocultivation test. The relationship of protein concentration to 2-anthramine activation by rabbit alveolar macrophage S-9 and rat liver S-9 was analysed. Protein dose curves were constructed using a set concentration of 2-anthramine. Rat liver S-9 showed optimal numbers of revertant colonies per plate at 0.5mg S-9 protein/plate. RAM S-9 showed increasing numbers of revertants/plate with increasing protein concentrations up to 2mg/plate but no optimum number.

The ability of primary rat hepatocytes to metabolize benzo(a)pyrene B[a]P into nine individual metabolites was determined using an HPLC procedure developed in this laboratory in order to assess the utilization of these cells for metabolic activation purposes. Hepatocytes in culture 1.5 and 24 hours were incubated with B[a]P for 4, 8, 12, and 24 hours. In general the longer the incubation time the less metabolites were formed per cell (probably due to the toxic nature of some of the B[a]P metabolites). Conversion to B[a]P water soluble metabolites also increased markedly with the longer incubation times. 1.5 hour old hepatocytes were more effective (38 pmoles metabolites formed/hr/million cells) in oxidizing B[a]P to organic soluble metabolites than were 24 hour old hepatocytes based on an 8 hour incubation time (15 pmoles metabolites formed/hr/million cells). Six mutagens were tested in the hepatocyte mediated _S. typhimurium_ suspension test at two concentrations of cells and 4 doses of chemical. 2-aminoanthracene, 2-aminofluorene, and aflatoxin-B gave positive results and dose-response; acetylaminofluorene was weakly positive, while B[a]P was negative with Ta1538. Dimethylaminoazobenzene gave no effect with TA98.

Bibliography

None

Related Research

The effect of whole animal exposure to acid mists and particulates on the pulmonary metabolism of benzo(a)pyrene in the isolated perfused lung model is being studied at the University of Cincinnati. The isolated perfused lung (IPL) appears to be the best *in vivo* preparation for investigating pulmonary metabolism of foreign compounds especially compounds adsorbed onto particulate. An important aspect of current work is the assessment of the rate of formation and types of metabolites formed when B[a]P is administered with ferric oxide or crude air particulate (CAP) on the IPL.

The isolated lung perfusion technique was used to study in an "*in vivo*" situation, the effects of SO_2, crude air particulate (CAP), and benzo(a)pyrene treatment on benzo(a)pyrene metabolism. The influences of these agents on the metabolism of benzo(a)pyrene, an environmental carcinogen, may explain differences in the carcinogenicity of this agent. SO_2, CAP and B[a]P pretreatment increased B[a]P metabolism in the IPL and increased the formation of specific activated metabolites, indicating that these agents may have cocarcinogenic activities.

SECTION IV

EVALUATION OF HAZARDS TO MAN

A. TASK TITLE:

Effect of Coal Gassification Products on the Pulmonary Defense System Against Infectious Disease

HERL/RTP TASK NO.: 8162

GRANTEE: Catherine Aranyi

GRANT NO.: 805317

Summary

This is one of several tasks designed to investigate the effects of particulate effluents from energy processes on host defense mechanisms against infectious disease. In this particular task, mice will be exposed via inhalation to particulates from conventional and advanced coal power sources and challenged with viable microbes. Subsequent evaluation of the animals will measure resistance to the induced pneumonia. The total, as well as specific defense systems (i.e. pulmonary bactericidal activity and alveolar macrophages) will be examined. The experiments will be designed to elucidate dose-response relationships. Since the work began recently, results are too preliminary to report.

Scope and Objective

The objective is to determine the effects of particulate effluents from coal utilization processes on host defenses against pulmonary infectious disease. Several biological endpoints will be used to define the dose-response relationships for the aerosols to be tested, to establish the effects of duration of exposure (acute and intermittent), and to determine the time required for recovery from exposure. Depending upon the availability of particulate effluents, several samples will be tested, with the emphasis being placed on advanced coal conversion processes.

Background and Approach

The role of air pollutants as a predisposing factor to respiratory infection has been well recognized. Extensive work with O_3 and NO_2, and auto exhaust and limited work with selected metals and sulfates has indicated that the relationship of these pollutants to pulmonary infectious disease may have an impact on the public health. While many of these pollutants are known to be associated with energy-related effluents, no research has addressed the complex particulates emitted from these processes. Because of the wide range of individual compounds, often of unknown chemistry, emitted from energy-related processes, it was decided that a reasonable approach would be to study the effects of the actual particulate effluent. By using biological endpoints of proven sensitivity in environmental inhalation toxicology to study these effluents, it is thought that the data derived can be used to assist in the assessment of the health effects of these compounds.

The principal experimental approach will use the infectivity model. With this system, mice exposed to air or to various concentrations of particulates for various lengths of time are challenged with aerosols of viable microbes (bacteria or virus). The animals are then held in clean air for 15 days during which time mortality is recorded. Prior work with this model has indicated that its sensitivity is most probably due to its ability to reflect the net effect of subtle alterations in a number of host defense parameters. These parameters, which will also be investigated separately, include: pulmonary bactericidal activity, and changes in the number, distribution and viability of free pulmonary cells which can be obtained by lavage. Based upon the results of these studies, a decision will be made as to whether additional related endpoints should be added, such as functional and biochemical integrity of the alveolar macrophage, a cell primarily responsible for sterility of the deep lung.

Research Accomplished

To date, the major emphasis of this recently awarded contract has been to set up facilities and ensure that the model systems are working properly. Aerosol generation and monitoring techniques have been finalized, key personnel have been trained, and preliminary experiments have been conducted with most of the model systems to be used. Facilities for aerosolization of radiolabeled bacteria (necessary for the bactericidal studies) are being built, with no cost for capital equipment to EPA. When this facility is completed, the bactericidal technique will be modified and perfected for this particulate study.

Preliminary work has been conducted with acute exposure of mice to aerosols of the first sample, a size fractionated ($\leq 3\ \mu m$) particulate from an electrostatic precipitator from a conventional coal power source. This same sample has been sent to other researchers (Task Nos. 8163, 8149, 8173, 8198). The preliminary nature of the work precludes a report at this time.

Related Research

Task Nos. 8162, 8149, 8163, 8173, and 8198 are all related in that each is designed to study various aspects of the effects of actual particulates from energy sources on host defense mechanisms. It is intended that all the work will be conducted on the same particulate sample so that results of all the studies can be compared and used to develop a better assessment of effects. In isolated instances, sample availability problems may cause some deviations from this approach.

In addition, the particulate sample used in this study will also be used for Task No. 8317, entitled "Effect of Industrial Particulate Emissions on Alveolar Macrophage," which is designed to correlate the in vitro alveolar macrophage system with the infectivity model. Under this Air Health Task, the sample will be used in the in vitro portion of the study only, but results will be compared to the in vivo model of Task 8162. The sample will also be assessed under Task No. (Huisingh) for mutagenic and carcinogenic potential using in vitro screening systems.

Please see the individual task reports for details of these studies.

B. TASK TITLE:

To Determine Effects of Pollutants From Alternate Energy Sources on Pulmonary Antiviral Mechanisms

HERL/RTP TASK NO.: 8163

GRANTEE: Leonard Schiff

GRANT NO.: R-805049

Summary

This task is one of several which will compare the effects of particulates from conventional and advanced energy processes on host defense mechanisms. In this project, the structure and function of mucociliary transport processes which are involved in pulmonary clearance are the focus of investigation. The ability of the particulate-exposed tracheal tissue to respond to viral infection will also be examined. Initially since particulate samples were unavailable, a study was conducted with H_2SO_4 and carbon, alone and in combination. Currently, particulate exposures are being made.

Scope and Objective

The purpose of this task is to compare the effects of conventional and advanced coal utilization processes on tracheal mucociliary transport parameters. Both in vitro and in vivo inhalation exposures will be conducted. In this way the validity of the in vitro test can be assessed for suitability as a rapid screening model. Dose-response studies of acute and intermittent exposures will be conducted and recovery from any adverse effects of this treatment will be examined.

Background and Approach

Due to the proliferation of both conventional and advanced energy processes, it becomes increasingly important to examine any health effects that may result from fugitive emissions from these processes. Actual particulate effluent samples will be tested to gather information in a faster and more relevant manner than testing each chemical species of the complex particulate separately. Dose-response and recovery studies will be conducted.

Depending on particle size, many of the particulates will deposit on the ciliated epithelium of the lung. This surface is covered with mucus and is responsible for clearing the lung of inhaled matter (both viable and non-viable), thus protecting it from a variety of insults. A proven sensitive model for determination of effects on this pulmonary surface will be used and involves examination of ciliary beating frequency and the morphology of exposed isolated tracheal rings or explants. Cialiated epithelium also defends the host against viral infections so several related endpoints of this interaction will be examined also (viral replication, interferon production, and morphology).

Research Accomplished

Until actual effluent particulates were available, work was conducted using H_2SO_4 (1.1 mg/m^3) and carbon (1.5 mg/m^3) alone and in combination, a model type pollutant exposure relevant to energy processes. Immediately after a 3 hr. exposure, there was a significant depression in ciliary beating in both the acid and acid-carbon exposure groups. Beating was depressed for up to 24 hrs. after exposure to the acid-carbon treatment and up to 72 hrs. after exposure to acid. In vitro exposure to these pollutants also caused decreased ciliary activity.

Immediately after exposure to the acid-carbon mixture, the tracheal epithelium showed greater morphological alteration than when exposed to air, carbon or acid mist alone. The examination involved light and scanning electron microscopic techniques.

In addition to refining methods for viral exposure and measurement of viral effects, recent work has focused on the effects of in vitro exposure (1 hr/day, 5 days/wk for 10 days) of hamster trachea to particulates ($\leq 3\ \mu$m) from an electrostatic precipitator of a conventional coal-burning power source. At $\geq$100 g/ml there was a depression in ciliary beating frequency after the first exposure. At $\geq 50\ \mu$g/ml, morphological alterations were observed. Tracheal explants exposed to 10 μg/ml fly ash for 3 hr/day experienced a decrease in ciliary beating frequency by day 8 and cytotoxic and histological effects at day 7. Work in progress includes combination exposures to virus and particulate. In vivo particulate exposures are scheduled to begin shortly.

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Related Research

Task Nos. 8162, 8149, 8163, 8173, and 8198 are all related in that each is designed to study various aspects of the effects of actual particulates from energy sources on host defense mechanisms. It is intended that all the work will be conducted on the same particulate sample so that results of all the studies can be compared and used to develop better assessment of effects. In isolated instances, sample availability problems may cause some deviations from this approach.

In addition, the particulate sample used in this study will also be used for Task No. 8317, entitled "Effect of Industrial Particulate Emissions on Alveolar Macrophage," which is designed to correlate the in vitro alveolar macrophage system with the infectivity model. Under this Air Health Task, the sample will be used in the in vitro portion of the study only, but results will be compared to the in vivo model of Task 8162. The sample will also be assessed under Task No. (Huisingh) for mutagenic and carcinogenic potential using in vitro screening systems.

Please see the individual task reports for details of these studies.

C. TASK TITLE:

Quality Control for Assessment of Human Exposure

HERL/RTP TASK NO.:	8164
CONTRACTOR:	Northrop Services, Inc.
CONTRACT NO.:	68-02-2788

Summary

This task was established to insure that the Clinical Environmental Laboratory and the Mobile Clinical Laboratories located in Chapel Hill, N. C. are operated according to predetermined standards of performance with respect to safety and data validation. The purpose of these laboratories is to assess the effects of exposure to common air pollutants on human test subjects. There are humanitarian and legal ramifications associated with the use of human beings as test objects. The safety and well being of both the people being tested, and the people conducting the test, is of primary importance. It goes without saying that every precaution must be taken to verify personnel safety from overexposure to pollutants or electrical accidents.

The data collected at this facility will be used to establish clean air standards, and will almost certainly be challenged. In view of the possibility of accident or challenge to the data, every step practicable must be taken to insure that the laboratory operates according to predetermined standards of performance with respect to safety and data validity.

The range of activities designed to accomplish this has been termed Quality Assurance (QA). A contract was awarded to Northrop Services, Inc.

on September 30, 1977 to design, develop and implement performance audits on the experimental systems used in these laboratories. The audits will include:

1. examination of operations and documentation
2. comparison of actual operations and established requirements
3. recommendations for remedial and preventive action
4. independent measurement of performance

The Clinical Environmental Laboratory is identified by the acronym CLEANS for Clinical Laboratory for Evaluation and Assessment of Noxious Substances. CLEANS is a system of two environmentally controlled clinical laboratories where human subjects can be housed for periods of time ranging from hours to weeks. Precisely controlled concentrations of gaseous and water soluble particulate pollutants, found in the environment, can be introduced, either singly or in combinations, into the clinical exposure laboratories. In addition, to the pollutants, the temperature, humidity, lighting, and air flow simulate natural ambient conditions. While in the controlled environment, the test subjects will undergo a regularly scheduled program of noninvasive pulmonary function tests, exercise electrocardiography, and systolic time interval measurements, along with physical examination and biological specimen collection. All aspects of these tests as well as the operation of the Clinical Laboratory are controlled and recorded by dedicated computers and peripheral equipment.

The Mobile Clinical Laboratories are identified by the acronym CLEVER for Clinical Laboratory for Evaluation and Validation of Epidemiologic Research. CLEVER consits of two forward control bus-type motor vehicles that house mobile clinical laboratories that contain the same or similar medical examination equipment, dedicated computers, and peripheral equipment as the CLEANS clinical laboratories. Where the CLEANS system was designed to measure and process noninvasive human physiological parameters in a controlled environment, the CLEVER systems were designed to make the same physiological measurements in the uncontrolled outside ambient environment. The normal home base for the CLEVER vehicles is at the Chapel Hill Clinical Studies Division. If the vehicles are in use at another location when scheduled for a QA audit, the Contractor is expected to transport the necessary equipment and personnel to that location to perform the required audit.

Scope and Objectives

The objective of this task is to obtain expertise from a Contractor to conduct independent Quality Assurance audits of all components of the CLEANS/CLEVER Systems which may affect the assessment of human exposures. In addition to his expertise, the contractor will use the "Quality Assurance Handbook for Air Pollution Measurements Systems" and the "Health Effects Research Laboratory Quality Assurance Manuals" as guides in developing a comprehensive Quality Assurance (QA) program that will cover all of the appropriate QA elements and responsibilities outlined in these documents. The main thrust of the QA program is:

1. To conduct a System Survey Audit to gather information on the current operations of the laboratories, to evaluate the Quality Control procedures now being used and to identify major problem areas.
2. To design verifiable audit test procedures and develop an auditors' handbook incorporating the QA program and test procedures.
3. To conduct periodic performance audits of the chambers and laboratory systems.

Background and Approach

A Request for Proposal (RFP) to develop and implement a comphrehensive Quality Assurance Program for complex and diversified experimental systems used to measure the effects of air pollutants on humans was published on May 27, 1977. The RFP No. DU-77-B154 was sent to thirty-one potential contractors, but only one responded with a proposal. The cost proposal was several times the amount budgeted for this project. Consequently, a revised proposal was requested from the contractor during negotiations. The cost of the revised proposal was within the budgeted funds for the project and was technically acceptable. The contractor had simply overestimated the project Scope of Work in his original proposal.

A contract was awarded to Northrop Services, Inc. on September 30, 1977.

A comprehensive quality assurance program is equally important to the quality control procedures that are an integral part of any responsible research protocol. The QA program corroborates the experimental results produced by the laboratory by providing a means for independently assessing and documenting that the experimental data has precision, accuracy, validity, is representative of the condition being measured, and is complete. A QA program also provides a means to evaluate the relevance of a task to laboratory and agency objectives.

The data obtained from exposing human subjects to various air pollutants in the CLEANS chambers will be used to support Federal Standards for limits of exposure. A well designed and implemented QA program is intrinsic to an experimental system that must produce data that is supportable and allows comparability among groups.

Accomplishments

Since the award of the QA contract to Northrop Services, Inc. the following tasks have been completed:

1. Original Work Plan describing the various tasks, the approach to be used to meet the objectives, and the projected schedule of the activities, was submitted to the Project Officer on October 28, 1977.
2. A System Survey of the CLEANS System Operation and Maintenance was completed February 24, 1978.

3. Monthly Progress Reports have been issued since the inception of the QA contract starting with the period October 1, 1977 through October 31, 1977.

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Related Research

This task directly supports the research tasks of the CLEANS/CLEVER experimental systems by providing the total Quality Assurance program required by the Health Effects Research Laboratory/RTP, N.C., management policy. Personnel of the Clinical Studies Division, HERL, are responsible for the development and implementation of exposure research protocols using the CLEANS/CLEVER system supported by the Operation and Maintenance Contractor, Rockwell International Corp. The objectives of the CLEANS/CLEVER research protocols are to determine and measure the health effects of humans from the exposure of gaseous and inhaleable particulate matter found in the emissions from alternate energy sources such as low grades of coal. The purpose of this research is to provide information necessary to formulate environmental regulatory policies to protect or improve public health and welfare while at the same time enhancing the nation's continuing productivity. This project requires periodic independent QA performance audits.

D. TASK TITLE:

Evaluation of the Hazards of Exposure to Biological Active Agents Associated with Energy Technology

HERL/RTP TASK NO: 8168

CONTRACTOR: University of Illinois

CONTRACT NO: 68-02-2492

Summary

A "Model for Measuring the Health Impact From Changing Levels of Air Pollution" is a federally funded EPA contract with the University of Illinois, School of Public Health. The award was originated February 15, 1977 with a proposed deadline of May 15, 1978. A time extension was sought for a new deadline of October 15, 1978. The study involves creation of a model to quantitate the relationship between ambient air concentrations of various individual air contaminants and health in a large metropolitan area in the Midwest.

The study encompasses both a mortality and morbidity component. The mortality component involves examination of residents of the city of Chicago whose deaths occurred between January 1, 1971 and December 31, 1975, and were registered in a respiratory or cardiovascular category (8th Revised I.C.D.). By comparing an individual's place of residence within the boundries of the city with normally occurring and periodic levels of air pollution, the contribution of certain pollutant parameters to these deaths may be ascertained and quantified. Climatological indices as well as socio-economic variables are accounted for in a statistical equation. In the morbidity component illnesses from respiratory or cardiovascular causes is used as an index of health.

For one year, April 1977 until April 1978, visits by residents of Chicago to two city hospital emergency rooms were monitored. Each resident's address was cross-referenced to one of seventy-six community areas designated by the U. S. Census Bureau. Contaminant levels in a specific location and various health effects are quantified.

Scope and Overall Objectives

The overall objective of this continuing research is to examine the strength of the relationship between concentrations of various ambient air contaminants and health indices. Mortality rates and emergency room visitation rates for cardiac, vascular and respiratory diseases in an urban, industrial, energy intensive area are being examined.

The following questions are addressed and answered in the stated research:

1. How much does each air pollutant contribute to cardiac and respiratory disease mortality?

2. How much does each air pollutant contribute to the incidence of cardiac and respiratory disease?

3. Are the national primary ambient air quality standards appropriate for the protection of public health?

Multiple regression analysis plays a vital role in finding each pollutant's contribution to mortality and morbidity in tandem with description statistics and the analysis of variance. Regression analysis describes the collective and separate contribution of two or more independent variables (pollutants, socio-economic variables, or climatological factors in the study) to the variation of a dependent variable (mortality index or morbidity index).

To statistically test the hypothesis that air pollution affects health, it is important to rule out the possibility of spurious correlations between some other variables and both air pollution and higher mortality rates. For example, there are several reasons why the mortality rate is higher in an urbanized area than in a less urbanized area (e.g. tension, stress, unhealthy personal habits). Thus, an urbanized and industrial area contributes simultaneously to air pollution and health status, and there is the possibility that any correlation between air pollution and health status would be spurious for this reason.

We would propose four factors affecting health status in a geographical area:

1. demographic characteristics - age, sex, race distributions
2. socio-economic characteristics - income distribution, housing density, occupation, and education levels.
3. environmental factors - air pollution levels, climatological characteristics, and energy utilization factors (heating fuels, etc.)
4. personal factors - smoking habits, medical care (quality and quantity) exercise habits, nutrition and genetic characteristics.

These factors are not all included in the model. However, those demographic variables provided in the 1970 census reports will be utilized, as will all climatological variables and air pollution parameters, in the development of a mathematical equation.

Background and Approach

The literature has clearly identified a positive correlation between levels of air pollution and rates of illness and death. Examination of ambient air concentrations of various pollutants can determine which factors influence mortality and morbidity rates. Once the various influencing variables have been determined, it is possible, through statistical technique, to determine the degree of influence of each variable.

As the study is divided into two components, each shall be dealt with separately. The first section is a three-fold mortality analysis, being conducted to test the relationship between death and air pollution:

1. Descriptive trend analysis is being conducted to ascertain the mortality trend in Chicago and downstate Illinois. The following tasks have been completed:

   a. comparison between Chicago area death rates by cause, by age, and by race with those rates in downstate Illinois for the years 1967 to 1975;

   b. annual death rates for 1967 to 1975, from all causes save accidents, homicides, and suicides, for all ages and races, were compared among seven chosen communities of varying size and location throughout Illinois;

   c. using the same seven cities, annual air contaminant measurements (TSP and SO_2, where available) were examined and compared, thus delineating citywide annual trends for 1967 to 1975;

   d. standardized age-adjusted death rates in Chicago for all causes were computed for 1970 to 1975.

   e. annual TSP and SO_2 values have been charted from 1970 to 1975, at each monitoring station in the city of Chicago. Thus, this data may be directly compared with the trend of mortality data for the city.

2. The next technique is long term cross-sectional analysis. Based on ten leading causes of death (that is, excluding accidents, homicides, and suicides), age-adjusted death rates for 1970 to 1975 are computed for each of the seventy-six community areas. At the same time annual air pollutant measurements (TSP and SO_2) are computed for the same five year period at each of the thirty-five monitoring sites. Each site operated at varying time intervals. Air contaminant values are interpolated to each community area, based on location of the site. Each area is then assigned to one of three air pollution categories (high, medium, low) based on its air contaminant value. To determine each air pollutant's contribution to cardiac and respiratory disease mortality, multiple regression is applied. The dependent variables in the equation are the death rates, by cause, during the five year period from 1971 to 1975. Independent variables included the average concentration levels of air pollutants (TSP, SO_2, NO_2, NO, CO, and ozone), weather factors, and socio-economic indices, percent of families with income below poverty level, number of white collar workers, level of education, and breakdown of ethnic groups. (This information is ascertained from the 1970 Census Report). The proportion of each pollutant's contribution to the total variance of the death rate is calculated after controlling socio-economic indices.

3. A time/series analysis is used to examine more closely the acute health effects of variations in the concentrations of ambient air pollutants. Weekly, monthly, and seasonal analyses are conducted, based on the availability of air pollution data. The dependent

variable is death rate from cardiac and respiratory diseases (for the appropriate time period). All disease categorizations are in accordance with the 8th Revised International Classification of Disease specifications. Independent variables are the average exposure levels of the deceased to the air pollutants and averages of climatological indices, such as temperature, windspeed, humidity, and sunshine. The average exposure level corresponding to each death is determined by correlating the person's place of residence with the estimated pollution level in that area. The exposure levels of all deaths are combined, and then an average exposure level is obtained by dividing the sum of the individual exposure levels by the number of deaths on that day (week, etc.).

The morbidity component of this study attempts to establish a cause-effect relationship between air pollution levels in Chicago and an increase in the number of cardiac and respiratory illnesses. The sources of morbidity patient data include two hospitals, Cook County (CC) and University of Illinois (UI). Cook County was chosen because it was found in an earlier study that CC registered at least as many patients per week as 13 other hospitals in the Chicago area. Also, virtually all of the patients were residents of the Chicago area and most were residents from the Chicago inner city and used CC as a primary care facility.

The UI Hospital was selected because in 1977, while data collection was in its initial stages, a doctor's strike at CC prompted most of CC patients to be routed to UI. To be assured of the continuity and availability of all necessary data, it was considered safer to continue monitoring both hospitals.

Within these hospitals, data are collected from three areas; the Emergency Room, Admissions and Pediatrics. Information is sought from these areas because the largest majority of these study subjects are non-appointment patients who need medical attention for acute care. Another very significant reason is that data obtained from these patients can be considered highly reliable because a doctor's diagnosis accompanies each patient registered and treated.

Data are collected by trained personnel Tuesday, Wednesday, and Thursday of every week. By omitting collection on weekends, days surrounding weekends (Fridays and Mondays), and holidays, a fairly regular pattern of admissions is established.

A standard form, entitled Hospital Record of Patients with Respiratory and Cardiac Conditions, is filled out to record information on all persons whose illness is applicable to the study. The following information is gathered on each form:

1. Sequence/number

2. Date

3. Time of Visit

4. Patient's Address

5. Patient's Age

6. Patient's Race

7. Patient's Sex

8. Patient's Complaint

9. Physician's Diagnosis

In addition to the patient data, other information is also collected. Total number of persons seeking medical help is recorded daily for each hospital area. A tally is kept of all patients who are pre-admissions, transfers, referrals, rechecks, refills, or dead-on-arrival (DOA'S). This number is subtracted from the total and the resulting number of study subjects is designated as the disease population number.

The forms are then number coded for community area, census tract, patient's complaint and physician's diagnosis. After the sequence number is stamped on each form, the forms are checked and sent out to be keypunched. Keypunched cards are listed and checked against the data forms for possible errors, then data forms, cards and lists are filed for later analysis.

Research Accomplished

Mortality--

To date, progress has been limited due to unforeseen major problems in collecting and processing air pollution data, as cited below. Preliminary trend analysis has been completed, verifying a downward trend in mortality, as well as in air pollution measurements in Chicago.

Cross-sectional analysis and time/series analysis have been delayed until recently, due to unforeseen problems in cleaning, processing and organiaing a master file for daily pollutant averages.

The air monitoring network based in Chicago is a complex one. A wide variety of air contaminants are monitored including total suspended particulate, sulfur dioxide, nitrogen dioxide, carbon monoxide, nitric oxide, oxidants, methane, hydrocarbons and various metals. The network itself is comprised of 37 sites under the sanction of the city of Chicago Department of Environmental Control, the U.S. EPA, the State of Illinois EPA, the Department of Air Pollution Control, the Department of Commerce or the U.S. Public Health Service. Each pollutant is monitored by one of four different instruments. The time interval between collection periods at any one monitoring site may be 1, 2, 4, 8, or 24 hours, or 3 or 6 days.

Because the variety of contaminants, systems and collection periods is so wide, a petition was made to the U. S. EPA for a complete data set, on tape, which would contain daily continuous levels of particulate, SO_2, NO_2, NO, CO, O_x and COH for all monitoring stations, regardless of controlling agency, located within the city's limits.

The tape received contained three files: the first contained continuous TSP, SO_2 and NO_2 data from 1970 to 1976; the second, hourly SO_2, NO, NO_2, O_3 and CO data for 1970 to 1973; with the third covering the years 1974-1976 for the same contaminants. (At the same time, a petition was made to both the city agency (DEC) and the U. S. EPA for printed copies of data in their files. After projecting much data manipulation (time being spent to clean and organize this master daily file), it was felt that any error found might be due to excessive handling by the project data processors. Both agencies complied and provided such a listing of data.

First, this tape was dumped, to ascertain exactly what material was located therein. Separate files were made for each pollutant in the primary task of calculating daily averages for each site over the entire 5 year time frame (January 1, 1971 to December 31, 1975). In cleaning these files, careful precautions had to be taken to account for all monitoring specificiations, day, site location and code, monitoring agency, duration, type of instrumentation, and units of computation, in order to provide an accurate comprehensive listing of all air pollutant data.

As the tape figures were checked against those published by the city, many inaccuracies were found. Unknown instrumentation codes were listed, as were two values for one site (with all coding intact) at one time, missing values (for as long as eight months), stations miscoded to mimic the same values of another site, extra values not found on any other record and two copies of all the same parameters reporting different values.

Approximately eight months were spent adjusting and correcting this data by the supplementation of additional values, deletions to the file, or replacement with a new file entirely.

Cross-sectional analysis is about to begin. After computing annual means for each site with no missing values for each parameter, an air pollution field has been determined through interpolation to each community area. Descriptive analysis should be completed within the next quarter.

Morbidity--

Since the start of hospital data collection on 12 April 1977, numerous questions and problems have surfaced. Problems of interpreting hospital records and selecting applicable information decreased considerably after the collectors became more familiar with the total process.

In December 1977, the data from CC pediatrics was cancelled. The log records available to us could not guarantee even a small degree of reliability because the doctors' diagnoses were not available.

The last day of data collection will be 14 April 1978. At this point, we project that by 16 June 1978 the collection data will have been taken through the final states of completion (i.e. processed, cleaned and on tape) and will be ready for final analysis. This mass of data will be collapsed into large categories of total numbers for sex and age, race, complaints, diagnoses, etc. The complaints and diagnoses will be further subdivided into major respiratory and cardiac illnesses and cross-referenced, as previously described, to air pollution values. As in the mortality section, morbidity data will be analyzed by the use of multiple regression analysis and analysis of co-variance.

E. TASK TITLE:

Studies of Health Effects Resulting From Increased Indoor Air Pollution From Energy Conservation

HERL/RTP TASK NO: 8169

CONTRACTOR: Geomet, Inc.

CONTRACT NO.: 68-02-2294

Summary

By May 31, 1978 the contractor will have completed a 27-month effort to develop a tool for measuring indoor air pollution exposure. The major activity of the project consisted of extensive indoor/outdoor air pollution monitoring. These monitoring measurements comprised the basis for developing mathematical models for predicting indoor air quality from pollutant levels recorded at community monitoring stations where outdoor ambient air quality levels were measured.

Scope and Objectives

The objectives of the contract were to:

1. Review and assess indoor air pollution literature
2. Conduct indoor/outdoor air pollution monitoring
3. Develop mathematical models to predict indoor air quality
4. Develop a mobility/health document

Background and Approach

Very little information exists which relates indoor air pollution levels to the many factors that affect such levels. Elevated levels of specific pollutants have been measured in indoor environments, but little effort has been expended to determine the relationship between indoor levels/outdoor levels, indoor levels/indoor source strength, indoor levels/energy conservation measures, and indoor levels/house activities. It is necessary to know these relationships in order to be able to make decisions ranging from health effects of indoor pollution to the consequences of making a building energy efficient.

The goal of this project was to be able to estimate the indoor pollution based on known factors such as outdoor pollution levels, indoor sources and source strengths, house activity, and energy conservation measures. Taking all of these inputs, the contractor had to develop a model or series of models that could be used to predict indoor pollution levels.

In order to be able to determine what parameters were most important to indoor levels and how they interacted, a monitoring program was established. Houses of varying designs, locations, and pollution exposure were each monitored continuously for a two week period. This information was used first to develop the required relationships and then to verify the accuracy of the model. To verify that the model was equally applicable during all seasons, return visits were made to several houses during different heating/cooling periods.

Research Accomplished

Preliminary results indicate the model is most accurate for the less reactive pollutants. It also seems to work equally well in both hot and cold seasons.

During the monitoring period no violation of the ambient air standards was observed inside a dwelling. However, under certain combinations of events, the model would indicate there could be very high levels of indoor pollution. Inside concentrations levels of two pollutants were particularly noteworthy. Carbon dioxide was observed at over three times the ambient level, and very high levels of aldehydes were observed. The elevated CO_2 occurred when several people were present in a room at the same time. Aldehydes were observed in most buildings, but were particularly high in the mobile homes.

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1. The status of Indoor Air Pollution Research - 1976 (published)

2. Survey of Indoor Air Quality Health Criteria and Standards (published).

3. The Geomet Indoor-Outdoor Air Pollution Model: A Scientific Report (in clearance process)

4. Final Contract Report Phase 2 (submitted for review and approval) "Indoor Air Pollution in the Residential Environment". Volume 1: Data Collection, Analysis and Interpretation; Volume 2: Prototype Epidemiological Study; Volume 3: Supportive Documents.

Related Research

EPA's Health Effects Research Laboratory at the Research Triangle Park has under contract a projected title: "The Acute Respiratory Disease Study". It is a 12-month study to measure the incidence and severity of acute respiratory disease symptoms related to total subject exposure: exposure within the home and home neighborhood, exposure while traveling to and from work, and exposure in the work environment.

The U. S. Department of Energy is presently investigating the relationship of energy conservation measures in MED (minimum energy dwelling) houses to pollution levels found inside the buildings. The project title is "Air Quality Measurement in Energy-Efficient Buildings". This is a two-year project and is concerned with schools, hospitals, and other buildings of this type.

The American Society of Heating, Refrigeration and Air Conditioning Engineers (ASHRAE) has several committees devoting time to the indoor/outdoor pollution problem as affected by various heating and air conditioning measures. This organization is supporting a project of the Department of Mechanical Engineering at Iowa State University entitled "Experimental Energy-Conserving Building". This continuing project includes constructing a house that will be fully instrumented for measuring pollution levels and energy consumption parameters.

F. TASK TITLE:

Chemical Repository for Alternate Energy Source Materials

HERL/RTP TASK NO.: 8170

CONTRACTOR: Oak Ridge National Laboratory

CONTRACT NO.: ERDA(DOE) No. 40-60176 EPA No. IAG-D7-0129

Summary

This project provides physical and chemical support to health-effects research addressing alternate fossil energy technologies. Samples provided by the EPA and those acquired by the repository staff are made available to qualified researchers. Samples of particular interest to the EPA are characterized in terms of physical properties, elemental composition, and organic chemical composition. Biological evaluations of highest priority samples are supported through the preparation, including physical or chemical separation, of materials for biotesting and the identification of constituents responsible for observed bioactivities. Coal liquefaction, gasification, and combustion and oil shale processing are currently emphasized. The facilities may be used to archive samples of particular interest to the EPA or to acquire analytical characterizations of related materials undergiong study in environmental and/or health assessments of alternative fossil fuels technologies. Results of chemical and biological studies are forwarded to those contributing samples to the respository.

Scope and Objectives

The objective of this project is to improve the cost-effectiveness of research on the health effects of synthetic fuels by increasing the availability of research materials, improving the transfer of data between technology developers and health effects researchers, and improving interlaboratory comparability through chemical characterization and bioassay materials preparation services. Biological studies at the Oak Ridge National Laboratory supported by Pass-Thru funds and studies elsewhere chosen by the EPA project officer currently receive priority attention.

Background and Approach

The development of bioassay methods and their application to health assessments of new fossil fuels technologies requires the availability of

3. A particle-sized fraction of ash from coal combustion has been prepared and distributed to EPA-specified bioassay contractors. The elemental composition of the ash has been determined; organic analyses are underway.

4. A comparison was made of available subcontractors for the size fractionation of bulk quantities of particulates for bioassay research. Small scale particulates size fractionation facilities were established in-house and materials were supplied to EPA researchers.

5. Methodologies made available to support bioassay research included (a) the analysis of particle size distribution (b) the chemical separation of syncrudes for biotesting (c) the glass capillary column gas chromatographic analysis of organic constituents (d) the separation of multialkylated derivatives of polycyclic aromatic hydrocarbons from parent and simple alkylated derivatives, and (3) the isolation of highly mutagenic azaarenes from syncrudes.

6. On-site observation and assistance in experimental design was provided for the sampling of an above ground oil shale retorting experiment for materials potentially available for inhalation exposure.

7. Assistance was provided for a joint Laramie Energy Research Center, Pittsburgh Energy Research Center, Oak Ridge National Laboratory study of the combustion charactersitics of shale-derived oils. Insufficient quantities of combustion particulates were available for collection for biological study. Emissions measurement results are being compiled.

8. Studies addressing the comparability of bacterial mutagenesis response with in vivo response following intratracheal instillation and the utility of bacterial mutagenesis assay for discriminating between shale-derived crude oils are underway. Repository staff are responsible for the preparation of materials under study.

Bibliography

This provides general assistance to EPA programs. Results are reported in informal documents to the EPA project officer. Six such "topical reports" addressing the current inventory of materials, analyses of specified samples and the results of requested studies have been issued in the past 12 months.

Metholologies specifically developed in support of biological experiments and the results of applying these methods are to be submitted for publications. Reports resulting from the use of materials now in the repository (not funded by the repository agreement) include:

Clark, B. R., N. A. Goeckner, and I. B. Rubin. 1978. Organic Chemical Characterization of a Crude Shale Oil. Submitted for presentation at Confab 78: Government, Industry and Academic Technical Conference on Fossil Fuel Chemistry and Energy. Saratoga, Wyoming, July 25 - 28.

Clark, B. R. and M. R. Guerin. 1976. Chemical Characterization and Monitoring Studies of Effluents from Emerging Fossil Fuel Processes. Presented at Air Pollution Control Association's Conference on Toxic Substances. Cambridge, Massachusetts, November 8.

Clark, B. R. and M. R. Guerin. 1977. Chemical Characterization of Organic Constituents in Oil Shale Materials. Second ERDA Meeting and Workshop on Oil Shale Environmental Research. Richland, Washington, November 1-2.

Clark, B. R., C. -h. Ho, and A. R. Jones. 1977. Chemical Class Fractionation of Fossil-Derived Materials for Biological Testing. Symposium on Analytical Chemistry of Tar Sands and Oil Shale. New Orleans, Louisana, March 20-25.

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Epler, J. L., F. W. Larimer, T. K. Rao, C. E. Nix, and T. Ho. 1978. Energy Related Pollutants in the Environment: The Use of Short-Term Tests for Mutagenicity in the Isolation and Identification of Biohazards. Presented at Higher Plant Systems as Monitors of Environmental Mutagens; Workshops cosponsored by the National Institute of Environmental Health Sciences, Department of Energy. Marineland, Florida, January 16-18.

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Related Research

1. Analytical Chemical Support of Synthetic Fuels-Related Bioassay Research (Department of Energy). Methods are developed and applied to the fractionation of synfuels-related materials in a manner suitable for biological testing. Collaborating biologists identify biologically active subfractions which are subsequently further fractionated in search of individual constituents responsible for biological response. Polycyclic aromatic hydrocarbon isolates from syncrudes have been isolated and characterized for mouse dermal bioassay.

2. Chemical Characterization of Synthetic Fuels (Department of Energy). Analytical methods are developed and applied to the characterization of organic constituents of synfuels and synfuel production waste streams. Phenolics, paraffins, bases, and polycyclic aromatic hydrocarbons have received primary attention.

3. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment (Department of Energy). In a collaborative project supported through the Environmental Sciences Division, multicomponent methods are being developed to isolate and quantify polycyclic aromatic

technology-derived materials for study. Perfectly representative samples do not exist because none of the technologies under study have achieved the state of development where commercial scale plants are operating. A mechanism was required which would provide research materials for bioassay methods development while maximizing feedback to the technology developers and minimizing premature extrapolations to overall technology assessments.

Environmental and health considerations are to be taken into account in identifying the most suitable synthetic fuels technologies. Chemical and physical analyses or treatments of materials required to support biological research are generally well beyond the scope of analyses required for process development. A mechanism was required whereby materials subjected to the most intensive biological study could be physio-chemically characterized to the degree required by the health effects research.

Some materials prove to be more useful than others in elucidating the biological characteristics of synthetic fuels technologies and in developing suitable bioassay systems. A mechanism was required to ensure the maximum utilization of these materials. Maximum utilization can only be achieved if the chemical and biological histories of the material are defined. Defined storage and chemical history also allows an assessment of the comparability of bioassay results between laboratories and of the degree to which bioassay results may be extrapolated to the technology as a whole.

These objectives are being met by combining the efforts underway within the Bio/Organic Analsis Section of the Analytical Chemistry Division, sponsored by the EPA, DOE, and EPRI, to the degree that joint interests allow. Research materials acquired in the execution of these programs are maintained within the repository and made available to all programs, subject to the approval of the technology developer supplying the sample and of the agency project officers involved. Measurement and sample preparation methodologies developed as results of these studies are transferred to the repository personnel for supporting EPA use of repository materials. Direct support of EPA health effects research is provided through input into design, preparation of materials for bioassay, characterization of materials undergoing bioassay, and assistance in acquiring sample materials of particular interest. A mechanism is being established to ensure that sample contributors are informed of physical, chemical, and biological data resulting from the use of samples provided.

Research and Services Accomplished

1. The repository currently contains 108 sample materials derived from five coal liquefaction or purification processes, two coal gasification processes, three shale oil recovery processes, and four coal combustion sources. Recent acquisitions include suites of samples from shale oil recovery operations in the USA and USSR. Material storage and inventory facilities are being upgraded.

2. A total of 110 samples have been distributed to 22 investigators in 32 requests.

hydrocarbons in stream water and sediment from the vicinity of a coal coking operation.

4. Polycyclic Organic Matter of Fly Ash (Electric Power Research Institute). Polycyclic organic matter adsorbed on fly ash is being compared with that found on electrostatic precipitator ash to determine whether bulk ashes can be utilized as surrogates for chemical study of fly ash and to determine the nature of organics available for ash pond leaching.

5. Inhalation Bioassay Monitoring (National Cancer Institute). Tobacco smoke inhalation bioassays carried out at other laboratories for the Smoking and Health Program are supported through the collection and analysis of samples taken at the bioassay laboratory, the development of monitoring and dosimetric methodologies and instrumentation, and general troubleshooting. Aerosols provided to the animals for inhalation by the various systems in use are chemically and physically characterized to define exposures.

6. Tobacco Smoke Analysis (National Cancer Institute). The smokes and smoke condensates produced by experimental cigarettes undergoing biololical study are characterized through the quantitative determination of particulate and vapor phase constituents. Special studies and analytical services are provided as required by the project officer.

G. TASK TITLE:

Preparation and Characterization of Fine Particulate Environmental Contaminants for Biological Experimentation.

HERL/RTP TASK NO.: 8171

CONTRACTOR: IIT Research Institute

CONTRACT NO.: 68-02-2451

Summary

Contract 68-02-2451 supports health effects studies conducted by EPA and by EPA contractors by providing preparation and characterization of particulate matter in the respirable size range. Several types of particles produced by energy-related processes have been provided. This contract terminated on March 31, 1978. Future plans for this type of contract depend completely on the plans for future health effects studies.

Scope and Objective

This contract was initiated for the purpose of providing well-characterized particulate matter resulting from various energy processes for use in *in vivo* and *in vitro* toxicity studies. Therefore, this contract functions in a supporting role to other contracts. The particulate matter supplied has included mineral fibers-amosite, fibrous amphiboles from the Peter Mitchell Pit (PMP) of Reserve Mining Company, and Cummingtonite-benzo(a)pyrene/lead oxide suspensions in gelatine-saline, and benzo(a)pyrene/ferric oxide combinations in a powdered state. The prepared materials are delivered to the EPA or to designated EPA contractors.

The aforementioned particulate matter is reduced in size if necessary into the respirable size range and then is characterized as to particle size distribution and chemical properties.

Background and Approach

This contract is essentially a continuation of similar work done by the same contractor on Contract No. 68-02-1687 which was a 3 year effort. The contractor served as a centralized source for preparation and characterization of selected particulate materials. As such the contractor developed much expertise in the area of mixing benzo(a)pyrene and metal oxides to produce either suspensions or dry powders for *in vitro* testing. Both ball milling and co-precipitation were examined and evaluated as methods for making these mixtures.

Work on mineral fibers was begun in the last year of Contract No. 68-02-1687. Fibers were extracted laboriously from taconite rock found in the Peter Mitchell Pit. These fibers were reduced in size to be comparable to samples found polluting the environment.

Research Accomplished

Fibrous amphiboles-both PMP fibers and UICC amosite-were supplied according to schedule for use in *in vitro* studies conducted by Northrop Services Inc. (NSI) Research Triangle Park, North Carolina. This preparation did involve a research effort as it had not been attempted previously. Ambient air samples were collected in areas of contamination. Fibers of similar chemical composition to those in the air samples were separated from the rocks containing them and then milled to produce a particle size distribution similar to that found in the air samples. These samples were supplied to NSI in a multitude of small sealed vials containing an inert atmosphere. Physical and chemical properties were determined. Standard UICC amosite was also supplied in the same manner, and its properties were also determined.

Suspensions of benzo(a)pyrene/metal oxide were prepared by ball-milling and supplied for carcinogenesis studies. For each batch that was supplied, a particle size distribution and the percentage of benzo(a)pyrene and metal oxide were determined.

A powder of benzo(a)pyrene in combination with ferric oxide was supplied to EPA for use in *in vitro* studies. Ferric oxide was sintered at different temperatures to obtain groups of particles having different surface areas; then the fraction of each batch between 1 and 5 μm aerodynamic diameter was separated. These three batches of ferric oxide having the same particle size range but different surface areas were then combined with benzo(a)pyrene by precipation of the organic compound from solution onto the ferric oxide. The particle size distribution of each batch and percentage ferric oxide and benzo(a)pyrene were determined.

Finally, work was conducted to set up a system for aerosol generation and monitoring of fly ash from a conventional power plant. This work was performed for an EPA Contractor.

The significance to the energy program of the above-described work is directly related to that of the health effects research projects utilizing the prepared particles and the generation systems.

Bibliography

As of yet there have been no publications resulting from this work other than the required quarterly reports.

H. TASK TITLE:

Effect of Material from Alternate Energy Sources on Whole Animal Defense Mechanisms

HERL/RTP TASK NO.: 8173

CONTRACTOR: Southwest Research Institute

CONTRACT NO.: 68-02-2286

Summary

This task is one of several which will investigate the effects of particulate effluents from conventional and advanced energy processes on host defense mechanisms. This project will utilize both *in vitro* and *in vivo* inhalation exposure models to assess effects on alveolar macrophages, the primary defense cell of the deep lung. By comparing the results of the *in vitro* exposure of guinea pig and baboon alveolar macrophage to the *in vivo* exposure of guinea pigs, it may be possible to predict the likelihood of using the *in vitro* system as a screening tool. The inhalation exposure will be used to determine dose-response effects and the presence of any delayed effects and recovery. Because of delays in acquiring particulate samples for testing, only preliminary tests have been conducted to date.

Scope and Objective

The purpose of this project is to compare the effects of conventional and advanced coal utilization processes on pulmonary disease, primarily infectious disease. Animals will be exposed acutely and intermittently to particulate effluents and examined immediately and at various times after exposure to allow observation of possible delayed effects or recovery. Various biological endpoints, including the alveolar macrophage, will be studied. *In vitro* exposures will also be conducted on alveolar macrophages from 2 animal species to permit correlations to validate possible *in vitro* models. Dose-response studies will be conducted.

Background and Approach

With increasing use of new, as well as conventional, energy technologies, it is important to define any possible association between particulate by-products of these processes and risk to the public health. Since these pollutants would most likely be delivered to man via inhalation, the lung is a possible target site. The alveolar macrophage which is primarily responsible for clearing debris and maintaining sterility of the gaseous exchange regions will be the focus of the experiments. This essential host-defense cell is susceptible to damage by O_3, NO_2, and metallic particles and will most likely phagocytize energy-related particulates, thereby increasing the likelihood of adverse effects. Histamine content of the lung will also be measured. If effects are found, this endpoint, which would be related to asthma, will be investigated further.

Guinea pigs will be exposed via inhaltion to various doses of the effluent sample. Alveolar macrophages from guinea pigs and baboons will be exposed *in vitro*. From the *in vivo* and *in vitro* exposures of guinea pig alveolar macrophages, it can be determined if the *in vitro* system is relevant. If it is, then by comparing the results of the guinea pig and baboon macrophages, improved predictions of possible human effects may be possible. In addition, if the *in vitro* testing is validated, future tasks could be designed for screening. Due to the large variety of particulate effluents and the difficulties in obtaining large quantities of sample, *in vitro* screening would be helpful in ranking samples for later *in vivo* testing.

Several functions of the alveolar macrophage will be investigated to permit better determination of the biological significance of any observed effects. These functions include: phagocytosis, response to lymphokines (macrophage migration inhibition factor which is involved in keeping the cell at the site of inflammation so that is may cause destruction of the cause of inflammation), tumoricidal capability, and bactericidal activity. Enzyme activities of the alveolar macrophage which relate to bactericidal activity and the cell's role in fibrosis will also be studied.

Research Accomplished

Due to the delay of EPA in obtaining particulates for testing, work was conducted at a very slow pace until recently and involved development of methodologies. Animals are now being exposed to particulate ($< 3\ \mu m$) from an electrostatic precipitator of a conventional power plant. Data are being collected on the parameters listed above, but, as yet, the number of animals tested is too small for accurate statistical analyses.

Related Research

Task Nos. 8162, 8149, 8163, 8173, and 8198 are all related in that each is designed to study various aspects of the effects of actual particulates from energy sources on host defense mechanisms. It is intended that all the work will be conducted on the same particulate sample so that results of all the studies can be compared and used to develop better assessment of effects. In isolated instances, sample availability problems may cause some deviations from this approach.

In addition, the particulate sample used in this study will also be used for Task No. 8317, entitled "Effect of Industrial Particulate Emissions on Alveolar Macrophage," which is designed to correlate the *in vitro* alveolar macrophage system with the infectivity model. Under this Air Health Task, the sample will be used in the *in vitro* portion of the study only, but results will be compared to the *in vivo* model of Task 8162. The sample will also be assessed under Task No. (Huisingh) for mutagenic and carcinogenic potential using *in vitro* screening systems.

Please see the individual task reports for details of these studies.

I. TASK TITLE:

Environmental Mutagens Studies Utilizing a Drosophila Test System

HERL/RTP TASK NO.: 8189

CONTRACTOR: Dr. John Baum

CONTRACT NO.: IAG D701207

Summary

The investigation will test the feasibility of adding the *Drosophila* system to the battery of tests presently utilized in the assessment of environmental mutagens. Current efforts are directed toward development of dose-response information and comparison of sensitivity of various wild type *Drosophila* strains.

Scope and Objectives

The initial objective of this research is to evaluate alternative approaches for adding *Drosophila* mutagenesis experiments to complement the battery of tests currently utilized to evaluate mutugenic effects of ambient air pollutants. This evaluation will proceed in the following steps:

1. Several wild type *Drosophila* strains will be tested for sensitivity (sex-linked recessive lethal test) to ethylene dibromide and other appropriate index mutagens. Experimentation will be conducted under a variety of dosage schedules to determine the feasibility of performing field experiments simultaneously and in the same mobile exposure facilities with *Tradescantia*.
2. If the above experimentation demonstrates that any strains studied are sufficiently sensitive to be appropriate for field tests, deploy the tests to approximately one site per month to test for mutagenic effects from direct exposure to ambient air pollutants.

3. If no suitable wild type strain is identified, an effort will be undertaken to identify other more sensitive, e.g. repair deficient strains. Upon demonstration that any such strain is suitable for deployment in the field, tests will be conducted as in (2) above.

4. If no strains are appropriate for field experiments, feeding experiments will be undertaken as follows: The material collected from high volume air samplers will be removed and mixed with *Drosophila* food. The *Drosophila* will be raised from egg stages to adult stages on this mixture. The effect of this treatment will be evaluated by sex-linked recessive lethal test.

5. An attempt will be made to develop and utilize a protocol to inject in *Drosophila* a portion of the material collected from the filters. The insects treated in this manner will be subjected to sex-linked recessive lethal test.

Background and Approach

For several years Brookhaven National Laboratory, through an agreement with National Institute for Environmental Health Sciences, has been working on the development of a *Tradescantia* test system which would be sensitive to air pollutants. Following successful experiments in controlled environments, a mobile facility was constructed which would allow testing under actual, as opposed to simulated environments. Because of the need for simultaneous air measurements during field tests, HERL was asked to collaborate.

Because of our continuing efforts to determine appropriate locations for epidemiologic studies of environmental carcinogenesis, it was decided that HERL should support the effort so that experiments could be conducted in areas under consideration for epidemiologic studies either because of high cancer rates or because of known or suspect carcinogens in environment.

In addition to the provision of a mobile van with a standard complement of aerometric equipment, HERL has developed methods for collection and transport of substantial quantities of both particulate and vapor phase pollutants for chemical analyses and for additional mutagenicity tests.

The battery of tests which are currently available for further assessment of these samples includes bacteria systems, tissue cultures and neoplastic transformation. Addition of the *Drosophila* system is desirable for several reasons: heritable rather than somatic mutations can be assessed, *Drosophila* can be placed in the exposure chambers and tested simultaneously with with the plants, *Drosophila* can carry out metabolic activation as it occurs in mammalian liver, and detailed analysis of the various types of genetic changes can be undertaken to further understand the mechanisms and possible relationships between mutagens, teratogens and carcinogens.

A close working relationship has been established between the two groups of geneticists who will be working on this project through a similar ongoing research effort supported by ERDA to study the mutagenic effects of magnetic fields in both *Tradescantia* and *Drosophila*. Provision of facilities acquired in the course of this ERDA supported research constitutes a substantial contribution to this agreement.

Research Accomplished

Dose-response studies which have been performed to date seem to indicate that the sensitivity of this system is inappropriate for deployment into the field. This finding would not preclude laboratory tests of concentrated pollutant samples. Tests of relative sensitivity of ten wild type strains do not provide a basis for selection.

Bibliography

No publications or presentations have been prepared to date as a result of this research.

Related Research

This research project is closely coordinated with the ongoing *Tradeescantia* studies involving Brookhaven National Laboratory, National Institute for Environmental Health Sciences and EPA. Samples of pollutants collected at the various study locations are provided to Dr. Michael Waters, ETD, HERL for further bioassay.

J. TASK TITLE:

To Evaluate Existing and Improved Methods for Sampling, Transport, Storage and Analysis of Biological Specimens Which Might Serve as Indicators of Contamination by Effluents from Energy Technologies

HERL/RTP/TASK NO.:	8194
CONTRACTOR:	National Bureau of Standards
CONTRACT NO.:	IAG-D5-0568

Summary

The Specimen Bank is a national program using a systematic approach and standardized protocols to collect, analyze and store environmental and biological samples and data to be used to assess the accumulation and movement of harmful chemicals in the biosphere.

The concepts of the National Environmental Specimen Bank (NESB), real time monitoring and retrospective analytical capabilities are derived from its dual function. First, representative portions of samples included in the bank would be analyzed at the time of their introduction to provide real time monitoring and evaluation of pollutant trends. Evaluation of these trends would serve as early warning sentinels, triggering proper control measures to halt rising human body burdens before irreversible damage could occur.

Second, a specimen bank would enable analytical scientists to use tomorrow's more sensitive and specific methods of chemical analysis on today's samples. The improved measurement methodology would enable health scientists to determine accurate levels for substances that would be either undetectable or poorly analyzed by today's less sensitive methodology. The existence of a specimen bank would provide the opportunity to determine what the body burden of newly recognized toxic substances was in the past and to determine if their levels had changed with time.

The methodology developed under this program is now at the stage where it should be field tested. A Specimen Bank Pilot Program is scheduled to start in FY'79.

Scope and Objectives

The intention of this research, in collaboration with the National Bureau of Standards (NBS), is to evaluate and develop, where necessary, methods for sampling, transport, storage and analysis of biological specimens which might serve as indicators of contaminants by effluents from energy technologies, industry, and agriculture.

Background and Approach

Industrialized nations are being constantly reminded about the potential dangers to human health and the environment by the ever increasing inflex of new man-made substances into our ecosystem, Kepon and PBB being recent examples. The U. S. EPA is especially aware of this situation and is presently studying the feasibility of establishing a program, the NESB, that would provide a formalized, systematic approach to assess the environmental impact of these substances at a national level.

The adverse effects of some environmental agents on human health are already well established, although for many diseases, an environmental etiology is only suspected, and, in the remaining cases, the cause is unknown. In order to assess the hazards of pollution for populations at risk, it is necessary to have a knowledge of the pollutant exposure. This exposure of human populations to environmental agents can be assessed by an integrated approach to monitoring. In this manner, the total exposure of a population at risk can be evaluated in terms of the relative contributions from each route of exposure. Once the relative contribution of each environmental pathway has been determined, routine monitoring, for assessment purposes, can be limited to the critical routes, making monitoring more cost-effective.

Environmental monitoring of pollutant levels (air, water, food etc.) provides one approach to the assessment of human exposure. Another approach is provided by biological monitoring; the determination of pollutant levels in human samples. The immediate advantage of biological monitoring compared to environmental monitoring is that, in principle, it makes it possible to obtain a direct measure of dose of exposure. It is through environmental and biological monitoring that information of the magnitude of pollutant exposure in man is obtained. That information must then be related to the health effects produced by the pollutant.

The NESB concept of a national program functioning under a formalized systematic approach is in sharp contrast to the many individual biological and environmental monitoring programs being conducted in this country. The salient feature of the NESB program is the generation of validated data and the ability to compare data from one region of the country with that of another. At present, with a myriad of sampling, analysis and storage protocols being used, validation and comparison of data is often impossible, making most data speculative at best.

EPA's NESB is a well defined system of collection, analysis and long-term storage of selected environmental samples, providing two important outputs. First, the analytical portion of the NESB will provide real time monitoring data for pollutant trend analysis. This data will provide information on the adequacy of our present control technology and criteria standards. In addition, the real time monitoring data locates potential pollutant "hot-spots", thus serving as an environmental alarm system. Second, the NESB will provide properly stored samples for retrospective analysis enabling health scientists to determine accurate levels of substances that would be either undetectable or poorly analyzed by today's less sensitive techniques.

Research Accomplished

The FY 78 funds have been used to partially fund an interagency agreement with the NBS for the development of state-of-the-art methodologies for sampling, collection, preparation, analysis, and storage of biological indicator specimens which reflect contamination by effluents from energy technologies and other sources. The ultimate goal of the project is the development of the NESB.

Methodology development has been undergoing extensive laboratory testing over the past three years. Accomplishments to date include: identification of sample container material, sample handling techniques, sample preparation for trace element analysis and standardization of analytical techniques for twelve trace elements. In addition, investigations are underway to study the effects and conditions of long-term storage on sample integrity. The scientific effort is at the point now where it is necessary to scale up the developed protocols from the "lab-bench" operation to a modified banking program--the Environmental Pilot Bank. The pilot bank effort would give the scientists actual working experience through all stages of the banking effort, including: (1) specimen collecting; (2) preparation; (3) analysis and; (4) storage. The pilot program would concentrate on a limited number of samples, collected, analyzed and stored in a central facility. The approach would allow for strict control and constant evaluation over all operational procedures. Problems encountered in any aspect of the program would undergo extensive review, detailing the extent of the deficiencies so that corrective measures may be initiated and validated.

The initiation of the Pilot Bank Program is scheduled for FY 79. During FY 78, the following tasks are scheduled:

1. Samples of human liver and marine mussel tissue will be collected and analyzed for trace elements, and stored in a low temperature facility. Evaluation of the homogenization and sample splitting techniques will be made. Sample subsets will be freeze-dried and checked for elemental losses and contamination.

2. Continuation of protocol development for sampling and storage techniques in human tissue, food grain, aquatic intergrator and air intergrator matrices.

3. Initiate studies for organic contaminants in the above mentioned matrices, including sampling, storage, and analysis protocol development.

4. Modification of NBS clean-room facility for Pilot Bank sample preparation and storage. Install ultra-low temperature storage equipment.

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Related Research

This project is a cost sharing exterprise in which the NBS participates in the funding. In addition, the EPA has a bilateral agreement with the Environmental Agency of the Federal Republic of Germany to jointly share in the scientific investigation, leading to the development of the NESB.

K. TASK TITLE:

Effect of Pollutants from Coal Burning and Coal Gasification on the Immune System

HERL/RTP TASK NO: 8198

CONTRACTOR: Pennsylvania State University
College Park, Pennsylvania 16802

CONTRACT NO: 68-02-2472

Summary

This task is one of several designed to investigate the effects of particulates from conventional and alternate energy sources on host defense systems. This particular task is focused on effects of chronic particulate exposure on the systemic and pulmonary immune system. To date, preliminary studies have been conducted on the effects of a continuous 56 day exposure to carbon, fly ash (bag-house, conventional power) and particulate from an electrostatic precipitator (conventional power). It would appear from preliminary tests that the humoral immune system is more affected than the cell mediated immune system. The differences between the effects of the 3 types of particulates could be due to differences in concentrations of exposure.

Scope and Objective

The objective of this task is to determine the effects of particulate effluents from conventional and alternate energy sources on the immune system. Mice will be chronically exposed to either particulate effluent, carbon or air prior to assay of the humoral and cell mediated immune system. Both the pulmonary and systemic immune system will be examined. The phagocytic activity of alveolar macrophages from the exposed animals will also be tested.

Background and Approach

Previously, it has been shown that chronic inhalation exposure to carbon can cause alterations of the immune system which is responsible for defending the host against infectious and neoplastic disease. If carbon can induce these changes, it would be prudent to determine if particulates from conventional or alternate energy processes exert a similar effect. It would also be helpful to regulatory decision making to compare the effects of carbon to energy-related particulates. Therefore, such studies are being conducted using the same model system used for the original work with carbon. Mice will be exposed chronically and examined periodically during exposure.

Parameters for cell mediated immunity include: mitogen-induced transformation of T cells from the spleen and mediastinal lymph nodes which drain the lungs, functioning of recognitive T cells using mixed lymphocyte culture techniques, and ability of T cells to kill tumor cells. For humoral immunity the following paramters will be examined: mitogen-induced transformation of β cells from the spleen and mediastinal lymph nodes, antibody production by lymphocytes from the spleen and mediastinal lymph nodes following aerosol immunization with bacteria, and circulating antibody titers after aerosol immunization. Alveolar macrophage phagocytic activity will also be investigated since it plays a role in pulmonary immunity.

Research Accomplished

Due to unavailability of an EPA-supplied particulate effluent, work was begun with carbon and fly ash from a bag house of a conventional power source. Recently, an EPA supplied particulate from an electrostatic precipitator of a conventional power plant has been used. Since exposures are for 56 days, only 2 replicates have been conducted and statistical analysis is not yet complete. Therefore, all results should be considered to be preliminary.

Since results are preliminary, for brevity, only approximate exposure concentration ranges will be given here. Some endpoints were examined on different tests, hence, the range of concentrations. The concentrations were as follows: Penn State-supplied fly ash: .66-93 mg/m^3 (< 2.1 μm); a total of 1.9-2.3 mg/m^3 (< 5 μm). EPA-supplied particulate, .37-.69 mg/m^3 (< 2.1 μm); a total of 1.1-3.1 mg/m^3 (<5 μm) carbon .84-1.5 mg/m^3 (< 2.1 μm); a total of 5.4-2.5 mg/m^3 (< 5 μm). Generally, exposures caused few effects on cell-mediated immunity. However, for the number of antibody producing spleen cells after aerosol bacterial immunization, carbon caused a depression which was observed at days 7-56. The Penn State-supplied fly ash caused a reduction at day 21 only and the EPA-supplied particulate caused no effects after 35 or 56 days of exposure. Mediastinal lumph nodes did not appear to be affected by any of the treatments. Serum antibody titers were reduced after 7, 21, and 56 days of carbon exposure. The Penn State supplied fly ash was effective at 21 and 56 days, but the EPA supplied fly ash only caused depression after 56 days of exposure. Mitogen-induced transformation of B and T cells did not appear to be afftected by 35 days of exposure to carbon or Penn State-supplied fly ash. EPA-supplied particulate caused no effects after 1 week of exposure. Results of the studies on alveolar macrophages and lymphocyte tumoricidal capability are too preliminary to report at this time.

Related Research

Task Nos. 8162, 8149, 8163, 8173, and 8198 are all related in that each is designed to study various aspects of the effects of actual particulates from energy sources on host defense mechanisms. It is intended that all the work will be conducted on the same particulte sample so that results of all the studies can be compared and used to develop better assessment of effects. In isolated instances, sample availablility problems may cause some deviations from this approach.

In addition, the particulate sample used in this study will also be used for Task No. 8317, entitled "Effect of Industrial Particulate Emissions on Alveolar Macrophage," which is designed to correlate the *in vitro* alveolar macrophage system with the infecticity model. Under this Air Health Task, the sample will be used in the *in vitro* portion of the study only, but results will be compared to the *in vivo* model of Task 8162. The sample will also be assessed under Task No (Huisingh) for mutagenic and carcinogenic potential using *in vitro* screening systems.

Please see the individual task reports for details of these studies.

SECTION 5

OTHER CATEGORY PROJECTS

Title	Status*
The Pharmacodynamics of Certain Endogenous Mammalian Antioxidants During NO_2 Exposure Contractor: Stanford Research Institute Contract No. 68-02-1713 HERL/RTP Task No.	A
Human Biochemical and Physiological Response to Acute Photochemical Air Pollution Exposure Contractor: Copley International Contract No. 68-02-1724 HERL/RTP Task No. 8175	A
Chromosomal Aberrations in Peripheral Lymphocytes of Students Exposed to Air Pollutants Contractor: University of Utah Contract No. 68-02-1730 HERL/RTP Task No.	A
Nitrogen Dioxide Trends in Selected Chattanooga Communities Contractor: Research Triangle Institute Contract No. 68-02-1737 HERL/RTP Task No.	A
Chromosomal Abnormalities Associated with Known Low Level Occupational Ozone Exposure in Welders Contractor: Columbia University Contract No. 68-02-1738 HERL/RTP Task No.	
The Operation and Maintenance of Controlled Environmental Labs (CEL) and Mobile Physiologic Medical Vehicle (MPMV) CLEANS Contractor: Rockwell International Contract No. 68-02-2485 HERL/RTP Task No.	C

Determine Relative Irritant Potency of (a) Particulates Resulting from Oxidation to Sulfur Oxide, (b) Inert Particles Interacting with Sulfur Oxide. A
Grantee: Harvard School of Public Health
Grant No. RO-802030
HERL/RTP Task. No. 6307

Evaluate Effects of Chronic or Intermittent Exposure to Respirable Particles and Mists Using Mouse Pulmonary Infectivity Model
Contractor: IIT Research Institute
Contract No. 68-02-1717
HERL/RTP Task No. 6711

Cytotoxicity Evaluation of Selected Sulfates and of Source and Ambient Air Samples A
Contractor: Northrop Services
Contract No. 68-02-1567
HERL/RTP Task No. 6713 and 5101

Implementation of Screening Tests for Potentially Hazardous Airborne Particulate Material A
Contractor: Northrop Services
Contract No. 68-02-1566
HERL/RTP Task No. 8150

Workshop on Screening Systems for Alternate Energy Sources A
Contractor: Kappa Systems
Contract No. 68-02-2435
HERL/RTP Task No. 8154

Operation and Maintenance of Community Health Air Monitoring Program to Quantitate Air Pollution Exposure in Selected Health Study Areas C
Contractor: Xonics, Inc.
Contract No. 68-02-2493
HERL/RTP Task No. 7167

Interactions of Various Pollutants on Causation of Pulmonary Disease A
Contractor: IIT Research Institute
Contract No. 68-02-2274
HERL/RTP Task No. 7174

Human Biochemical and Physiological Response to Acute Photochemical Air Pollution Exposure
Contractor: Copley International
Contract No. 68-02-1724
HERL/RTP Task No. 8175

Field Testing--Application of Improved Combustion C
Technology to Power Generation Combustion Systems
Contractor: Exxon
Contract No. 68-02-1415
HERL/RTP Task No. 7184

Calibrate Cyclone Used to Obtain Large Quantities of C
Size Separated Particulate
Contractor: IERL (Piggyback)
Contract No. 68-02-2131
HERL/RTP Task No. 7186

Chemical Characterization and Specimen Preparation of B
Fibrous Amphiboles and Refined Particles
Contractor: GCA
Contract No. 68-02-2771
HERL/RTP Task No. 8188

Furnish Rabbits B
Contractor: Pel-Freeze BioAnimals
Contract No. 68-02-1638
HERL/RTP Task No.

Assessment of the Postnatal Development and Function of A
the Central Nervous System of Monkeys Exposed to Tritiated
Water from Conception to Birth or Weaning
Contractor: SRI
Contract No. 68-02-2280
HERL/RTP Task No.

Investigate Neoplastic and Life Span Effects on Potentially D
Sensitive Populations of Rats Chronically Exposed to Tritiated
Water
Contractor: Dawson Research Corporation
Contract No. 68-02-2289
HERL/RTP Task No.

Addition of Mobile Air Monitoring Field Stations and Portable A
Air Pollution Monitors to CHAMP System
Contractor: Rockwell International
Contract No. 68-02-0759
HERL/RTP Task No.

Operation, Calibration and Maintenance of Total CHAMP C
System
Contractor: Rockwell International
Contract No. 68-02-1745
HERL/RTP Task No.

Effects of NO_2 on Lung Function in Human Subjects with Asthma and Chronic Bronchitis A
Contractor: University of Maryland
Contract No. 68-02-1745
HERL/RTP Task No.

Stationary and Mobile Facilities for Study of Health Effects of Environmental Contaminants (CLEANS/CLEVER) B
Contractor: Computer Sciences
Contract No. 68-02-0768
HERL/RTP Task No.

Scanning Electron Microscopic Examination of the Effects of Air Pollutants on Pulmonary Systems A
Contractor: IIT Research Institute
Contract No. 68-02-0761
HERL/RTP Task No.

Preparation and Characterization of Fine Particulate Environmental Contaminants for Biological Experiments A
Contractor: IIT Research Institute
Contract No. 68-02-1687
HERL/RTP Task No.

Addition of Equipment for Generation and Monitoring Aerosols in the CLEANS Clinical Exposure Chambers B
Contractor: Environmental Research Tech
Contract No. 68-02-2300
HERL/RTP Task No.

The Effects of Low Level NO_2, O_3, and Ambient Air, Separately and in Combination, on Cardiac, Pulmonary and Peripheral Circulatory Functions in Adult Males, in Response to Heat Stress at Rest and During Moderate Exercise A
Contractor: University of California
Contract No. 68-02-1723
HERL/RTP Task No.

Collection and Characterization of Naturally Occurring Airborne Particulates A
Contractor: NBS
EPA IAG D4-F531
HERL/RTP Task No.

Operation and Maintenance of CLEANS C
Contractor: Rockwell International
Contract No. 68-02-2485
HERL/RTP Task No.

Human Biochemical and Physiologic Response to Acute Photochemical Air Pollution Exposure A
Contractor: Copley International Corporation
Contract No. 68-02-1724
HERL/RTP Task No. 8175